Mycobacterium. tuberculosis induces selective upregulation of Toll-like receptors in the mononuclear leukocytes of patients with active pulmonary tuberculosis

Thesis submitted for the degree of Doctor of Philosophy in Microbiological Immunity,
University of London

Jung-Su Chang (Susan, 張榮素)

Centre for Infectious Diseases & International Health
(Windeyer Institute of Medical Sciences)
University College London (UCL)

Janurary 2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Toll like receptors (TLRs) belong to the pattern recognition receptor (PRR) family which plays an important role in innate immunity by recognising conserved pathogen associated-molecular patterns (PAMPs) present on a wide range of microbes. In general, TLR2 (as a homo- or heterodimer with TLR 1 or 6) recognises Gram (+) bacteria whilst TLR4 recognises Gram (-) bacteria. The aim of this project was to investigate the role of TLRs in innate immunity in response to TB.

We investigated TLR gene expression in fresh unstimulated blood and bronchoalveolar lavage (BAL) from patients with pulmonary tuberculosis, using a well-validated real time PCR. A splice variant of TLR1, designated hsTLR1, was found in all donors tested. hsTLR1 mRNA lacks exon 2, which is a 77 bp region of the 5′ untranslated region, but contains the same coding sequence as TLR1. Compared to the matched controls, whole blood from patients had increased levels of mRNA encoding TLR2, TLR1, hsTLR1, TLR6 and TLR4. By contrast, expression of these TLR was not increased in BAL. An increased level of hsTLR1 mRNA was found in both $CD3^-$ and in $CD4^+$ cells resulting in an increased ratio of hsTLR1 mRNA to TLR1 and to TLR6 mRNA. An *in vitro* study in THP1 cells suggested that this relative increase in hsTLR1 might be attributable to a direct effect of mycobacterial components because it could be mimicked by mycobacterial preparations in the absence of IFN-γ or T cells, and by the TLR1/2 agonist, Pam3CysK4. Half-life studies using blood from TB patients and THP1 cells exposed to Mtb showed p38 MAPK-independent stabilisation of mRNAs encoding hsTLR1 and TLR1. We conclude that *M. tuberculosis* exerts direct effects on patterns of TLR expression, partly via changes in mRNA half-life. The significance of these changes in the pathogenesis of disease deserves further investigation.

Table of contents

Chapter 2. Materials and Methods

Chapter 3: Validation of Real time RT-PCR for gene quantification

Chapter 4. Expression profile of Toll-like receptors in patients with active pulmonary tuberculosis

Chapter 8. General Discussion

List of figures

Chapter 8

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 8

Dedication

~ 'To my parents who show me the strength, integrity and persistency in dealing with challenges. This work was made possible by the generous financial support of my parents and sisters. I am also in debted to almighty budda who gave me the strength and wisdom to finish this project.'

~ This thesis has no correction and this is attributed to the hard work of my principle supervisor, Prof. Graham Rook.

～ 榮素隨筆於中華民國 95 年 1 月, 英國倫敦

Acknowledgements

This project has proved to be tougher than I expected. The success of this project is mainly attributed to my primary supervisor Prof. Graham Rook for his ingenious guidance as well as financial support (SR Pharma). I am also grateful to Professor Ali Zumla for kindly having taken me into his group during my difficult times and my second supervisor, Dr. Jim Huggett, for his tireless support in molecular biology part of the project as well as general supervision. I am in debted to Dr. Helen Donoghue for advice on various aspects of my study in UCL and Dr. Louise Kim for sharing her expertise in immunology. A large part of this project would not have been possible without the generosity of Dr. Keertan Dheda, who has kindly provided invaluable clinical materials and kept me thinking straight at times of trouble. No words can describe my gratitude to Drs. Louise Kim, Jim Huggett and Keertan Dheda, who have kindly taken care of me when I was undergoing treatment of pulmonary tuberculosis. Finally, this thesis may well never have been completed without the exceptional support and understanding of my friends, Dr. Shijin He, Dr. Steve Evans, Rachel O'Mahoney, Fatima Miranda, Alan Pittman and Dr. Rohen de Silva, to whom I shall always be indebted.

I wish I have gained a little wisdom from this journey and am able to deal with next challenge with more patient and gratitude.

List of publications

- **Jung-Su Chang**, Jim F Huggett, Keertan Dheda, Louise U Kim, Alimuddin Zumla and Graham AW Rook. *M. tuberculosis* induces selective upregulation of Toll-like receptors in the mononuclear leukocytes of patients with active pulmonary tuberculosis. **J Immunol** 2006; 176(5):3010-8.

- **Jung-Su Chang**, Keertan Dheda, Jim F Huggett, Louise U Kim, Alimuddin Zumla and Graham AW Rook. Expression of IL-4 mRNA in peripheral blood mononuclear cells from normal donors in relation to expression of TLR2. **Immunology Letters** 2006; revised.

- Dheda K, Huggett JF, **Chang JS**, et al. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. **Anal Biochem** 2005; 344:141-3.

- **Chang J-S**, Huggett J, Kim L, Dheda K, Zumla A, Rook G (2004). Use of quantitative real-time RT-PCR to measure innate immunity. Presented Poster at 1st Real Time PCR conference, Munich, Germany.

Abbreviations

ActD (actinomycin D)

APC (antigen presenting cell)

AraLAM (ara-lipoarabinomannan)

AU (adenylate-uridylate)

BAL (bronchoalveolar lavage)

CCR (chemokine receptor)

CD1 (cluster of differentiation 1)

CDS (coding sequences)

CGD (chronic granulomatous disease)

CFP-10 (culture filtrate protein 10)

CMI (cell mediated immunity)

CpG-ODN (CpG-oligodeoxynucleotide)

CTL (cytotoxic T lymphocyte)

DC (dendritic cell)

DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin)

DMSO (dimethyl sulphoxide)

DN T cell (double negative T cell)

dsRNA (double-stranded RNA)

DTH (delayed type hypersensitivity)

dNTPs (deoxynucleotide triphosphates)

ERK (extracellular signal regulated kinase)

ESAT-6 (early secreted antigenic target 6 kDa protein)

LAM (lipoarabinomannan)

LTBI (latent tuberculosis infection)

MALP-2 (mycoplasmal lipopeptide)

MAPK (mitogen activated protein kinase)

MDR-TB (multi-drug resistant tuberculosis

MHC (histocompatibility complex)

Mtb (mycobacterium tuberculosis)

MTBC (Mtb complex)

MyD88 (myeloid differentiation factor 88)

MyD88s (spliced variant of MyD 88)

sMtb (M.tb sonicate)

sMV (*M. vaccae* sonicate)

SNP (single nucleotide polymorphism)

NOD (nucleotide-binding oligomerization domain)

Nramp 1 (natural resistance associated macrophage protein)

O_2^- (superoxide anion)

OH (hydroxyl radical)

PAMP (pattern recognition receptor)

PBMC (peripheral blood mononuclear cell)

PBS (phosphate buffered saline)

PDC (plasmacytoid DC)

ROI (reactive oxygen intermediates)

FACS (fluorescence activated cell sorter)

FITC (fluorescein-isothiocyanate)

GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

GF (growth Factor)

HIV (human immunodeficiency virus)

HLA (human leucocyte antigen)

Hsp (heat shock protein)

HPRT (hypoxanthine ribosyl transferase)

HuPO (human acidic ribosomal protein)

H2O2 (hydrogen peroxide)

hsTLR1 (human splice variant TLR1)

IDO (indoleamine 2,3-dioxygenase)

IL- (interleukin)

IL-4δ2 (interleukin 4 delta 2)

IRF-3 (interferon regulatory factor 3)

iNOS (inducible nitric oxide synthase)

INH (isoniazid)

iNK T cell (invariant natural killer cell)

Ipr1 (intracellular pathogen resistance 1)

LRR (leucine rich repeat motif)

LPS (lipopolysaccharide)

LTA (lipoteichoic acid)

PE (phycoerythrin)

PGN (peptidoglycan)

PHA (phytohemaglutinin)

RNI (reactive nitrogen intermediates)

RFLP (restriction fragment length polymorphism)

RPLP0 (60S acidic ribosomal protein)

RPMI (Rosewall Park Memorial Institute)

RT-PCR (reverse transcription polymer chain reaction)

sIL-4R (soluble IL-4 receptor)

sTLR2 (soluble TLR2)

smTLR4 (splice variant mouse TLR4)

SLE (systemic lupus erythematosus)

STK (Src-family tyrosine kinases)

TB (tuberculosis)

TGF (transforming growth factor)

Th (T-helper)

TIR domain (intracellular toll/interleukin-1 receptor domain)

TIRAP (TIR domain-containing adaptor protein)

TRAM (TRIF-related adaptor molecules)

TRIF (TIR domain-containing adaptor inducing IFN-β)

TLR (toll like receptor)

TM domain (transmembrane domain)

TNF (tumour necrosis factor)

TNFR (TNF receptor-associated factor)

PI3K (phosphotidyl inositol-3-kinase)

PMA (phorbol Myristate Acetate)

PPD (purified protein derivative)

PRR (pattern recognition receptors)

PTB (pulmonary tuberculosis)

TRAF (tumor necrosis Factor receptor-associated factor)

Treg (regulatory T cell)

TST (tuberculin skin test)

UTR (untranslated region)

Aims and Objectives

Tuberculosis is one of the most historical human infectious diseases. BCG vaccination was first administered to humans in 1921 and a short course chemotherapy regime was established by the British Medical Research Council (BMRC) in 1974 (Chapter 1, section 1.2 & 1.4.2). Yet, despite the advances in drugs and vaccination, the emergence of multi-drug resistant TB, human immunodeficiency virus (HIV) and the failure of control and treatment programmes, are leading to rising incidences of tuberculosis globally (discuss in Chapter 1, section 1.3). The optimum immune response against a pathogen requires the involvement of both innate and adaptive immunity (Chapter 1, section 2). The protective Th1 immune response (TNF-α, IFN-γ and IL-12) is thought to play a central role for controlling infection at an early stage (1-3). This has been established in various experimental models including human genetic studies, generating genetically defective mice and neutralisation of cytokines by drug administration in humans (Chapter 1, section 2.2 & 2.3). The fact that co-infection with HIV leads to reactivation of disease also strengthens the view that the protective immune response is mediated by effector T cells (4).

BCG vaccination, which boosts the Th1 immune response, fails to protect against adult forms of Mtb infection (5) and clinical studies reveal little evidence of defects in type 1 cytokines level (IFN-γ and IL-12) in TB patients (6-8). In fact, TNF-α, though it is essential in formation of tuberculosis granulomas, has been implicated in causing the immunopathology of tuberculosis (9-12). The immunopathology of tuberculosis is complex and several types of immune cells (APCs and T lymphocytes) and cytokines (Th1 and Th2) co-exist in granulomas (Chapter1, section 2.3). The existence of the Th2 cytokine IL-4 at the site of infection is gradually being acknowledged (6, 13, 14)

but it remains controversial whether that type-2 cytokines play a role in the immunopathology of tuberculosis (15). Nonetheless, this issue has provoked a debate about the immunopathology of tuberculosis and a call for new vaccine designs (16, 17). One hypothesis is that in susceptible individuals mycobacterial antigens induce a small but significant IL-4 response that undermines the immune system thus allowing *M. tuberculosis* to evade cellular mycobactericidal host responses (5, 16-18). Since only an estimated one in ten infected individuals develops disease (19), the interaction between host immunity and growth of the intracellular pathogen will be the key element that determines whether the outcome is elimination of the bacteria or latency or progressive disease.

Toll-like receptors (TLRs) act as bridge linking innate and adaptive immunity and play a centre role in host defence against microbial invasion (20). 11 members of the TLR family have been identified, and each member has characteristic of tissue distribution, ligand-specificity and control of gene-induction (Chapter 1, section 3.3) (21). Viable bacilli and components of Mtb are mainly recognised by TLR4 and TLR2, which is facilitated by heterodimerisation partners of TLR1/TLR6 (22-24). When triggered by appropriate ligands, TLR drive rapid cell activation, maturation and cytokine release (25, 26). This response plays a role in the initial innate response to infecting organisms, but also helps to determine the nature of the subsequently generated specific immune response (Chapter 1, section 3.2.3). Accordingly, gene defects in TLR2 or TLR4 are associated with susceptibility to microbial infection in both humans and mice (27-32).

Evidence from *in vitro* studies also suggests that *M. tuberculosis* may use TLR to subvert the host protective immune response (Chapter 1, section 3.5) (27, 33). This has

been mainly demonstrated *in vitro* by culturing cells with ligands of Mtb and observing subsequent cellular activation. This has led to two main findings explaining how Mtb is able to escape killing by the host:

1) mechanisms and properties of immuno-suppressive components of M.tb such as the 19-kD lipoprotein, 24-kDa lipoprotein (LprG) and ManLAM (34-36);
2) immuno-complexity (both Th1 and Th2) triggered by TLR ligands (37-40).

Firstly, the 19-kD lipoprotein inhibits phagosome maturation via the TLR2 pathway (34). The 19-kDa and LprG were reported to diminish T cell-mediated killing by altering antigen processing (36) or inhibiting maturation of APCs (35, 36). Treating DCs with ManLAM biased DC towards production of IL-10 instead of IL-12 (41). The view has been that a Th2 response, involving generation of T cells that secrete IL-4, IL-5 and IL-13, is the default, while responses generated in the presence of TLR agonists were more likely to be Th1-biased, and associated with IFN-γ production because of the tendency for TLR to drive IL-12 release (42).

Given the critical role of TLRs in innate immunity, and in the initiation of the appropriate adaptive response, the regulation of their expression is likely to be an important determinant of the clinical outcome of Mtb infection. The present study therefore sought to investigate the role of TLR in immunity to tuberculosis.

The current project can be divided into two sections. The first section examines the expression of TLR families in patients with active pulmonary disease compared to matched controls. The study of gene expression profiles requires cautious interpretation as shown in our previous study which suggested that M.tb treatment

selectively changes the stability of reference genes (45). Reference genes were therefore validated prior to the gene investigation experiment (46). Secondly, the thesis describes characterisation of the molecular biology of a novel splice variant of TLR1, designate hsTLR1, discovered during the project.

In summary, the aims of this project were:

- To set up a sensitive quantitative Real-time PCR assay and validate a suitable reference gene for the assay (Chapter 3). Because reproducible PCR assay is the key to ensuring successful gene manipulation, factors that affect reproducibility of PCR assay, such as primer concentration/annealing temperature, amplicon length, and amplification efficiency of standard plasmid, are carefully optimised. Since normalisation is required to see changes in expression of target gene, a suitable reference gene was optimised.

- To examine the expression profile of TLR mRNA in unstimulated peripheral blood, immune cell subsets and BAL from patients with active pulmonary tuberculosis (Chapter 4). The result of the clinical study was validated *in vitro* in THP1 cells treating with sonicated M.tb antigens as well as synthetic peptides.

- To characterise the molecular biology of a novel splice variant of TLR1, designated hsTLR1 (Chapter 5). The coding sequence of hsTLR1 was examined by cloning the full length transcript as well as it's RNA secondary structure by Mfold. The observation that Mtb-induced a relative increase in

mRNA encoding hsTLR prompted the investigation of mRNA stability and posttranscriptional regulation by p38 pathway.

- To examine the correlation between expression profiling of TLR and Th1/Th2 cytokine in peripheral blood of patients and control donors (Chapter 6).

- To conduct a pilot study to determine the expression profiling of TLR and host immune response to different *M. tuberculosis* strains (Chapter 7). The effect of strain-specificity on expression of TLR4, TLR2 and TLR2 co-receptors as well as Th1 (IFN-γ) and Th2 (IL-4) cytokine profiling was examined.

Chapter 1.Introduction

1. OVERVIEW OF TUBERCULOSIS

1.1. The origin of human tuberculosis: Genome analysis

Tuberculosis is one of the most historical human infectious diseases. Yet, in spite of its long association with mankind, knowledge of the *Mycobacterium tuberculosis* complex (MTBC) has only progressed in recent years when the genetic approach became available (47). Members of the *Mycobacterium tuberculosis* complex, the agents responsible for tuberculosis, compose a group of related species, such as *M. tuberculosis, M. bovis, M. microti, M. africanum, M. canettii and M. caprae,* despite differences in phenotypic characteristics, pathogenesis and mammalian host ranges (48, 49). The term "*M. tuberculosis* complex" distinguishes this group of organisms from saprophytic mycobacteria (e.g., soil organisms) and atypical mycobacteria such as *Mycobacterium avium–intracellulare* (50).

Because of the unusually high degree of conservation in their housekeeping genes (99% similarity), it has been suggested that the members of the MTBC underwent an evolutionary bottleneck at the time of speciation, estimated to have occurred roughly 15,000–20,000 years ago (51, 52). It was proposed that human tuberculosis evolved from *M. bovis* based on the assumption of cross-infection from different hosts (53). The whole genome sequence of *M. tuberculosis* (54) and comparative genomics (55) uncovered several variable genomic regions in members of the *M. tuberculosis* complex. Evidence from genome analysis suggested that based on the presence or absence of an *M. tuberculosis* specific deletion (TbD1), *M. tuberculosis* strains can be

divided into ancestral and modern strains (56). The modern strains, resulting from progressive gene deletion, are represented by the causes of major epidemics such as the Beijing, Haarlem, and African *M. tuberculosis* clusters. Authors suggested that the common ancestor of the tubercle bacilli might have resembled *M. canettii, and* could well have been a human pathogen already (56). A recent study by Gutierrez and colleagues (57) of human isolates of the tubercle bacillus, including *M. canettii* from East Africa, suggest a much broader progenitor species from which the MTBC evolved, estimated at 35,000 years old, as well as the ability of exchanging parts of their genome with other species. Thus, MTBC appears to have been coevolving with the human host longer than previously thought.

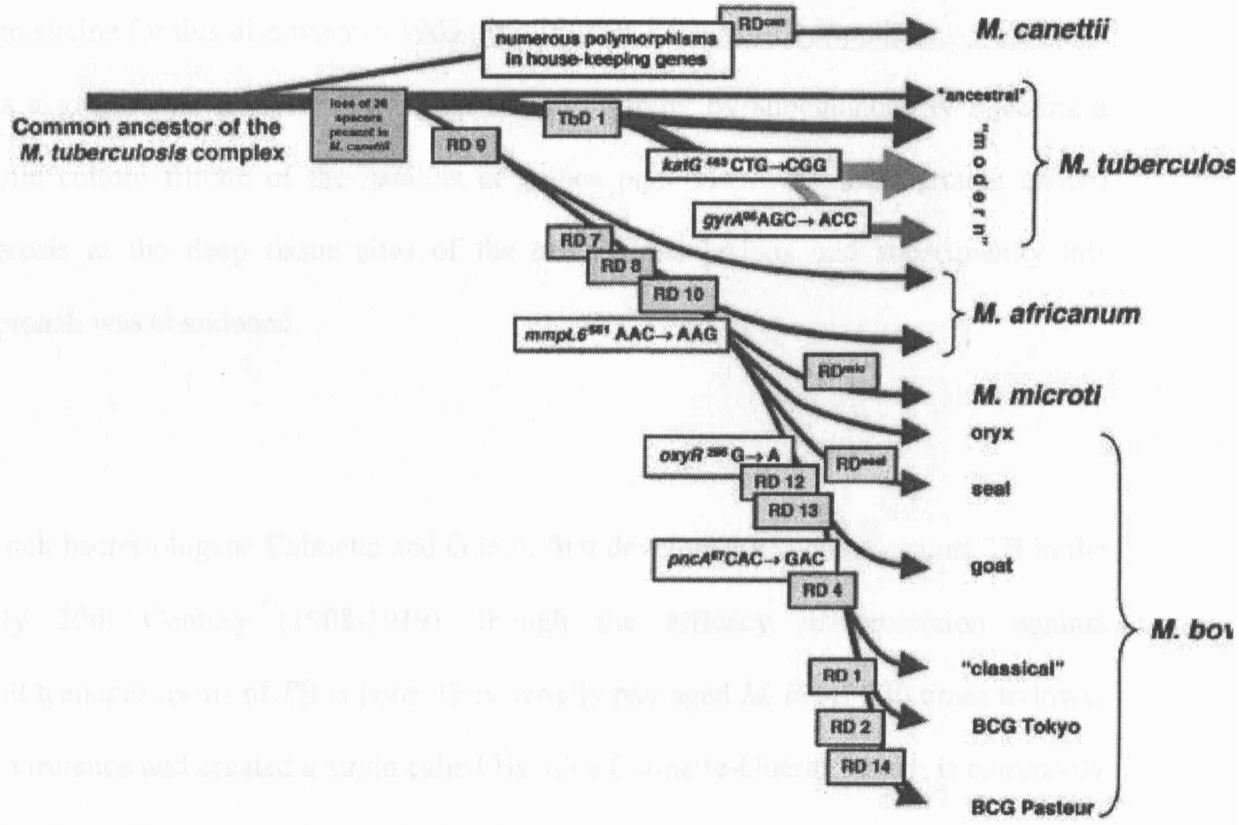


Figure 1.1. Schematic diagram of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes. Adapted from Brosch, B et al (2002) Proc Natl Acad Sci, 19; 99(6): 3684–3689.

1.2. Milestones in tuberculosis immunology

The term phthisis, consumption, appears first in Greek literature. Around 460 BCE, Hippocrates identified phthisis as the most widespread disease of the times, and noted that it was almost always fatal (http://www.umdnj.edu/~ntbcweb/history.htm). In 1720, the English physician Benjamin Marten was the first to suggest the notion that TB could be caused by "wonderfully minute living creatures" (Marten, 1720). In 1865, the French military doctor Jean-Antoine Villemin demonstrated that consumption could be passed from humans to cattle and from cattle to rabbits (Villemin, 1865). On the basis of this revolutionary evidence, he postulated a specific microorganism as the cause of the disease. In 1882, Robert Koch discovered a staining technique that enabled him to see *Mycobacterium tuberculosis*. He subsequently received the Nobel Prize in physiology or medicine for this discovery in 1905 (http://robert-koch.area51.ipupdater.com/). Koch was also the first to try experimental immunotherapy by subcutaneously injecting a sterile culture filtrate of the bacillus in guinea pigs. However, the injection caused necrosis at the deep tissue sites of the tuberculous lesions and subsequently this approach was abandoned.

French bacteriologists Calmette and Guérin first developed a vaccine against TB in the early 20th Century (1908-1919), though the efficacy in protection against adult/teenager forms of TB is poor. They serially passaged *M. bovis* 230 times to lower the virulence and created a strain called Bacillus Calmette-Guérin, which is commonly called BCG. BCG vaccination was first administered to humans in 1921, although the efficacy of BCG vaccination varies among the regions, it remains the standard vaccination today. Another important breakthrough during the 19 century was the realization of interaction of host immunity against TB infection. The role of

macrophages in host immunity against tuberculosis was appreciated by Metchnikoff (Metchnikoff, 1905) and Mackaness advanced knowledge on the role of lymphocytes in cellular immunity (Mackaness, 1968).

1.3. Epidemiology

Tuberculosis remains the world's leading cause of death from a single infectious disease. The World Health Organisation (WHO) reported that one third of the world's population is infected with *Mycobacterium tuberculosis* and estimated 9 million new cases each year worldwide (19). The major burden is in South-East Asia and sub-Saharan Africa, reaching 700 cases per 100,000 individuals in some areas (19). Although there has been a steadily declining prevalence of tuberculosis in the United Kingdom, USA and North East Asia since 1980, the prevalence of tuberculosis is rising again (58, 59). Between 1992 and 1998 the tuberculosis notification rate in London rose from 23 to 35 per 100 000, representing 41% of notifications in England and Wales in 1998 (60). In East Asia, the incidence rate of tuberculosis in 1996 was similar between Taiwan and Singapore, 54.06 (Centre for Disease Control, Taiwan, http://203.65.72.83/ch/dt/upload/qc/epi/epi_face.htm) and 57.2 (61) cases per 100,000 populations respectively. The prevalence of tuberculosis was higher in Hong-Kong (101.1 cases per 100,000) in 1996 (62).

However, recent reports reveal a trend of rising incidence in the East Asia region since 1996, even in the lowest prevalence country, such as Japan (59, 61-63). Alarmingly, the newly diagnosed cases increased significantly between 1996 and 2002 in Taiwan, from 54.06 to 74.6 per 100,000 (Centre for Disease Control, Taiwan, http://203.65.72.83/ch/dt/upload/qc/epi/epi_face.htm). Unlike in Africa and in South East Asia, the emergence of HIV infection contributes little to this rising trend in East Asia, though there is a tendency for the increase to occur among young males (age 20-40) (62-64). Factors, such as socio-economic decline during the Asian economic crisis in 1998, cross-border migration, increasing drug abuse, incomplete treatment (mainly incorrect prescribing of antibiotics) and deteriorating public health infra-structure account for this rise (65-67). The failure of effective treatment of tuberculosis also raises the issue of multiple drug resistant tuberculosis (MDR TB) (68) and tuberculosis in children (69). The WHO has now declared TB to be a 'global public health emergency' and has called for the urgent development of a new vaccine (5).

1.3.1. Incidence of Multiple Drug Resistant tuberculosis (MDR TB)

MDR TB is defined as resistance to Isoniazid (INH) and Rifampacin or more antituberculosis drugs. Two types of MDR TB exist: primary and acquired resistance. Primary resistance refers to persons who contracted a drug resistant strain of *M.tuberculosis*, whilst acquired resistance implies infection with a sensitive strain of *M. tuberculosis* that mutated to drug resistance inside the host, usually due to inappropriate treatment. A survey conducted by the WHO and the International Union against Tuberculosis and Lung Disease (IUATLD) in 35 geographic sites between

1996 to 1999 reveals a marked geographic difference in the prevalence of MDR TB (70). East Europe and South East Asian countries, such as Estonia (36.9%), Russia (32.4%), China (35% to 12.2% in different province) and Thailand (25.5%) had the highest prevalence of primary resistance cases. Acquired resistance occurred in all continents, with the highest incidence in Russia, Ivanova Oblast (68.5%), China, Henan Province (65%), Italy (60.6%), Estonia (59.85), Iran (57.1%) and India (50%). This survey suggested that South East Asia (China, India and Iran) and East Europe (Estonia, Russia and Latvia), other than Africa, had the highest prevalences of MDR TB among newly diagnosed cases. Interestingly, China showed a distinct geographic variation in the prevalence of drug resistance with a trend of association with each region's social economic status (68, 71). A marked geographic difference also exists in the prevalence of MDR TB in Taiwan ranging from Northern Taiwan (17.3%), Eastern Taiwan (7.5%) and Central Taiwan (3.9%) (68).

1.4. Transmission and latency

M. tuberculosis is usually transmitted by droplet spread. The 2 distinct phases of tuberculosis are those of infection and disease after first transmission. Evidence from laboratory animals has suggested that alveolar macrophages are the first cells infected by *M tuberculosis*. These infected macrophages are then surrounded by newly recruited macrophages and lymphocytes; together they form the tuberculous granuloma in the lung (3, 72). Bacterial latency is assumed to be induced by a protective Th1 response (TNF-α, IFN-γ and IL-12) in these tuberculous lesions. Therefore, the majority of infected individuals become latently infected (~90%), but is unable to eradicate the bacillus (73). Only an estimated one in ten (5-10%) infected

people develop active tuberculosis (74). The traditional view of *M. tuberculosis* dormancy was that it was exclusively confined within macrophages or macrophage-derived cell lineages *in vivo*. A recent study using IS6110 insertion sequence for the detection of mycobacterial DNA by *in situ* PCR in normal lung tissue revealed bacterial DNA in non-professional phagocytic cells (e.g. type II pneumocytes, endothelial and fibroblast cells) (75). When the balance between mycobacterial persistence and the host cellular response is disturbed (e.g stress, malnutrition, co-infection with HIV), reactivation may occur. Genetic predisposition may also contribute to the resistance and susceptibility (76). The estimated lifetime risk of developing reactivation in individuals with no HIV is approximately 10% (2 to 23%) (77).

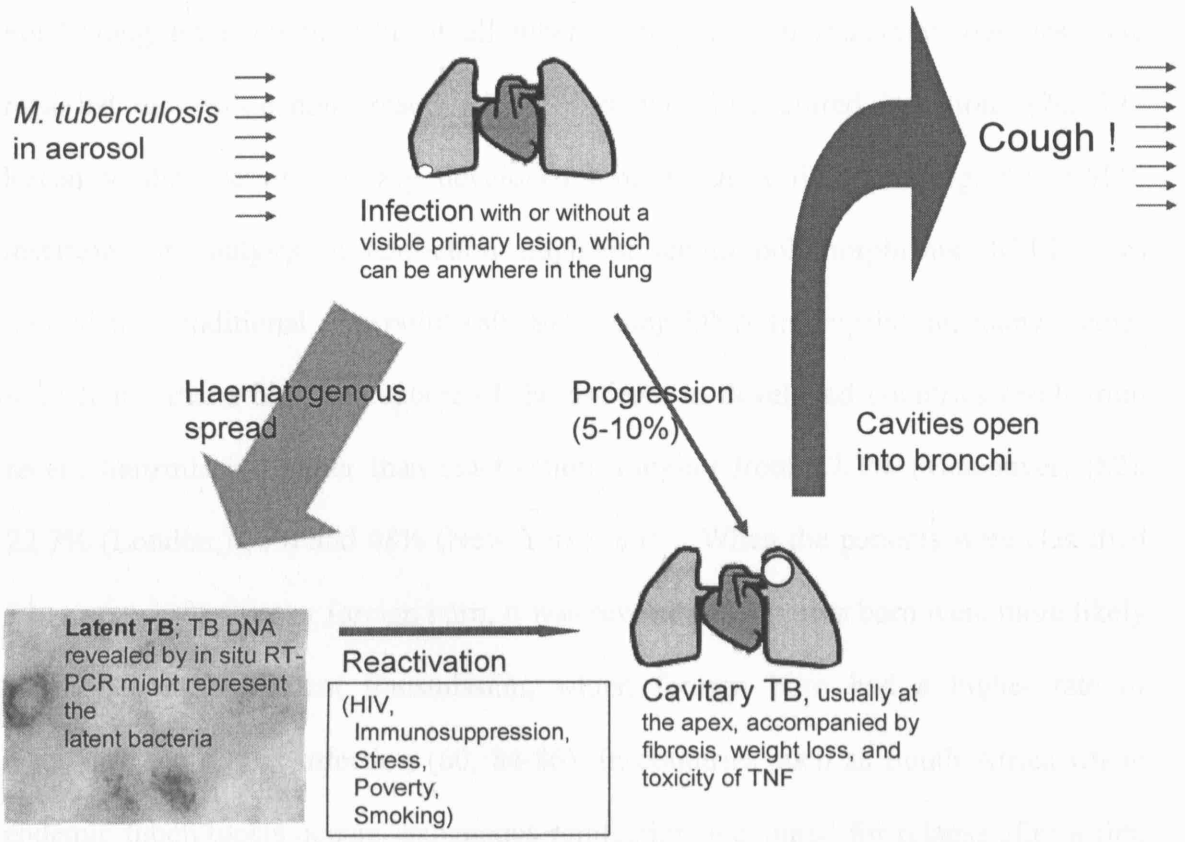


Figure 1.2. Illustration of phases of *M. tuberculosis* infection. Tuberculosis (TB) can establish infection with or without a visible primary lesion. This lesion can be anywhere in the lungs, but tends to be towards the base and close to the pleura. A T helper 1 (Th1)-cell response develops rapidly at the site of infection and in 90–95% of subjects, the infection remains latent. Bacterial DNA can be detected by *in situ* PCR (shown as blue intracytoplasmic material) in tissue with little cellular infiltrate. In a minority of individuals, progressive disease develops. In others, the disease may remain latent for decades but can reactivate when the patient is immunosuppressed. Cavities eventually open into the bronchi, allowing the spread of TB by aerosol during coughing.

1.4.1. Recent transmission versus Reactivation

For a long time, about 90% of all tuberculosis cases in industrial countries were regarded as "endogenous reactivation" of previously acquired infections (78, 79). Recently, the use of a newly developed tool of DNA fingerprinting, the IS6110 insertion for analysis of restriction fragment length polymorphisms (RFLP), has revised this traditional viewpoint (80, 81). Using DNA fingerprinting, many studies have found that a high percentage of the patients in developed countries result from recent transmission rather than reactivation, ranging from 17.3% (Vancouver) (82), 22.7% (London,) (60) and 48% (New York) (83). When the patients were classified into groups of native or foreign born, it was revealed that native born were more likely to be caused by recent transmission, whilst foreign born had a higher rate of reactivation of latent infection (60, 84-86). In countries such as South Africa where endemic tuberculosis occurs, exogenous reinfection accounted for relapse after a first successful treatment (12 out of 16 patients) (87) whilst endogenous reactivation was reported to be the major cause of relapse in UK (88) and The Netherlands (89). This assumes that clusters of patients belong to a chain of recent transmission and one index patient per cluster is regarded as endogenous reactivation from a primary infection (90). To clarify recent transmission versus reactivation in different regions is extremely important for planning effective treatment of the majority of latently infected individuals, thus preventing the disease spread.

1.4.2. Principle of TB treatment

Until the 1940s, there was no medication effective against TB in humans and the standard treatment included rest and fresh air. In 1944, first effective antituberculous drug, streptomycin, was introduced in the treatment of tuberculosis. Subsequently, a variety of antituberculous drugs was developed and a short course (six month) treatment regimen was established by the British Medical Research Council (BMRC) in trials in east Africa (91, 92). If administered appropriately, short course regimes, that combine Isoniazid (INH), Rifampicin, Pyrazinamide or Ethionamide, can cure more than 90% of pulmonary TB with a relapse rate less than 5% (91). The principle of successful of multiple medications for the treatment of tuberculosis was explained by Dr. D.A.Mitchison (93). He hypothesized that there are different populations of tuberculosis bacilli *in vivo* based on their metabolic activity and on their location: 1) actively metabolizing and rapidly growing, 2) semi-dormant in an acidic environment (e.g. in the early inflammation sites), 3) semi-dormant in non-acidic environment with occasional spurts of metabolism, and 4) bacilli in a dormant state. He suggested that Isoniazid is most active against the rapidly growing subpopulation and thus displays the highest acitivity among antituberculosis drugs, an effect known as early bactericidal activity. Drugs, such as Rifampicin and Pyrazinamide have long-term sterilizing activity which is believed to reduce relapse rates. Notably, no drugs are thought to be active against truly dormant bacilli. The most serious adverse effect is damage to the liver and potentially fatal hepatitis. The combination of INH and Rifampicin causes higher risks than either given alone because both of them are metabolised by the liver cytochrome p-450 oxidase system, which generates intermediate toxic metabolic products.

2. IMMUNOLOGY OF TUBERCULOSIS

2.1. Innate immunity

2.1.1. Pattern recognition receptors (PRR)

The vertebrate immune system is armed with innate and adaptive immunity. Innate immunity is the first line of host defense against microbial infection. This process is mediated by leukocytes (e.g. granulocytes, macrophage, and dendritic cells) which are in constant surveillance for invading organisms. The main role of innate immunity is to make an appropriate immune response according to the type of organisms and decide if it necessary to initiate adaptive immunity by presenting antigen to T cells. Innate immune cells might be able to distinguish self from non-self and to collect preliminary taxonomic information about microorganisms because they are equipped with a repertory receptors called pattern recognition receptors (PPRs), which by definition recognise conserved pathogen-associated molecular patterns (PAMPs) (e.g. orientation of certain sugar and lipid residues as well as spacing) (94, 95). The principle functions of PRRs include opsonization, activation of complement and proinflamantory signalling pathway, phagocytosis, as well as induction of apoptosis (96). The family of PRRs includes complement receptors, lectins, the mannose receptor, scavenger receptors, DC Sign and recently discovered NOD (nucleotide-binding oligomerization domain) and toll-like receptors (TLRs). The 'Danger hypothesis', proposed by Matzinger in 1994, is an alternative theory of explaining how the immune system reacts to endogenous antigens such as nucleotides, heat shock proteins (HSPs), reactive oxygen intermediates, synthetic peptides and inflammatory cytokines (97). Matzinger suggested that the immune system does not distinguish between self and non-self, but between dangerous and not dangerous. Studies of ligands recognised by TLR reveal that PPRs are able to recognise and initiate innate immune responses to endogenous antigens (e.g. HSP) as well as synthetic stimuli (e.g. Poly (I:C)) (21).

2.1.2. Bacterial killing by macrophages

Macrophages are professional antigen presenting cells (APCs) and play an essential role in both innate and adaptive immunity. There are two types of macrophages classified by their status of activation: the classical activation which is interferon-γ (IFN-γ) dependent and a so-called alternative activation by Interleukin-4 (IL-4) and IL-13 (98). The classical pathway of IFN-γ-dependent activation of macrophages is a well-established feature of cellular immunity to infection with intracellular pathogens, such as *M. tuberculosis* and HIV. IFN-γ is mainly produced by T helper 1 (Th1) cells (CD4+ and CD8+), NK T cells as well as macrophages themselves. IFN-γ is known for the induction of antimicrobial effector pathways, the up-regulation of macrophage cytokine production (e.g. IL-12, TNF, IL-6), the enhanced expression of class II MHC on APCs and the formation of granulomas in the early infection, which leads to the control of bacterial replication (99, 100). Other soluble mediators, such as IL-12, TNF and IFN-α/β are also known to promote macrophage function.

Mycobacterium tuberculosis (Mtb) is an intracellular bacterium that infects and replicates in macrophages. Following intracellular infection, Mtb resides in the phagosome where it is able to survive because it prevents acidification and phagolysosome maturation (101). Within the phagosome, Mtb also gains access to nutrient molecules such as iron via the early endocytic pathway (102). The innate immune response directed by activated macrophages dominates the initial host response to infection of Mtb but the optimum immune response requires the establishment of effector T cells. The classical IFN-γ-mediated activation of macrophages in mice probably leads to the killing of mycobacteria by the production of nitric oxide (NO). Catalytic action of the respiratory burst by the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex produces reactive oxygen

intermediates (ROI) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH^·$). NO is formed when the guanidino nitrogen of L-arginine is oxidized by an inducible nitric oxide synthase (iNOS). The antibacterial activity of NO and RNI was to modify bacterial DNA, protein, and lipids at both the microbial surface and intracellularly (103). Inhibition of NO production by iNOS inhibitors (aminoguanidine and NG-monomethyl-L-arginine), impaired bactericidal activity (104) and the subsequent control of latent infection (105) in a murine tuberculosis model. The important role of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in the controlling of *M. tuberculosis* growth was further confirmed in knockout mice (106). In a more recent study, depletion of CD4+ T cells in a mouse model of latent infection revealed that although CD4 was required for preventing reactivation disease, it was by an iNOS- and IFN-γ-independent antimycobacterial mechanism. These experimental findings indicate both NO-dependent and NO-independent mechanisms are operative to maintain the latent state (107).

Unlike the murine study, although human macrophages can express iNOS mRNA and protein on activation, the role of NO against mycobacteria in human remains controversial (103, 108, 109). Moreover, *in vitro* human models using either cell lines or macrophages induced from peripheral blood monocytes, require manipulation of the cells prior to experimental treatment. Factors, such as low detection level of iNOS and NO in human macrophages (110), differentiation status of macrophages (111, 112) and experimental design, e.g. mycobacterial strains (113), agents used for the *in vitro* differentiation of macrophages (114) and status of patients, e.g. MDR v.s normal infection (115) were reported to bias the results. The importance of ROI in antimicrobial defense is also exemplified in patients with chronic granulomatous disease (CGD), a disorder due to a mutation in any one of the four subunits of

NADPH-oxidase complex, resulting in an inability to generate ROI. In Hong Kong, the annual incidence of TB in patients with CGD was more than 170 times higher than in the general population (116).

Notably, another IFN-γ-inducible molecule involved in anti-microbial pathways, LRG-47, a GTPase that works independently of NOS2 was described by MacMicking *et al* (117). Mice lacking LRG-47 failed to control Mtb replication and defective bacterial killing in IFN-γ-activated LRG-47-/- macrophages was associated with impaired maturation of Mtb-containing phagosomes.

At a recent conference in Stockholm, on the pathogenesis of tuberculosis, Dr. Steffen Stenger provided some evidence that the anti-bacterial peptides, cathelicidins, might be important mediators of killing of *M. tuberculosis* by human macrophages (personal communication via Prof. G. Rook).

2.1.3. Dendritic cells (DCs)

Although both macrophages and DCs are professional antigen presenting cells (APCs), much of the attention is focussed on DCs in recent years because of their ability to drive naive T cells, and to influence their bias towards Th1, Th2, $Th_{IL\text{-}17}$ or regulatory phenotypes. Unlike macrophages, a professional phagocytic cell, the major function of DC is to take up antigen and activate naïve T cells. Accumulating evidence suggests that DCs play an important role in determining the type of immune response after presenting antigen to T cells (118-121). Several factors can influence the development of polarizing responses, such as DC lineage (118), activation status (120), antigen concentration (122), types of cytokine present (123) and DC:T cell ratio (124). DCs are able to recognise and up take M.tb components by DC-specific intracellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) or by TLRs (125, 126). Experiments using pCD11c-diphtheria toxin receptor (DTR)/GFP transgenic mice, which transiently delete DC *in vivo*, showed that DCs play a pivotal role in priming the $CD4^+$ T cell response following Mtb infection (127). Mtb can grow and replicate in both human peripheral blood-derived DCs and murine bone marrow-derived DCs, with a mature phenotype and inflammatory cytokine secretion (128, 129). Activation of bone marrow-derived DCs and macrophages with IFN-γ and LPS inhibited the growth of the intracellular *M. tuberculosis* in a nitric oxide synthase-dependent fashion but only activated macrophages are capable of bacterial killing (130). Constrained intracellular survival of *M. tuberculosis* in human monocyte-derived DCs was also observed by Tailleux *et al*, which was attributed to the impairment of mycobacterial phagosomes and the host-cell biosynthetic pathway, resulting in an inability to access essential nutrients (131).

2.2. T-cell mediated immunity

2.2.1. T cell lineage

During T cell development, a bone marrow-derived precursor undergoes three distinct cell fate decisions. First, a common lymphoid precursor, can differentiate to lymphocytes (T, B and NK cells), and must decide whether to adopt a T cell fate. Once the T cell lineage is specified, intrathymic T cell precursors ($CD44^+CD25^-CD117^+$) express CD25 and begin to rearrange and express their TCR β, γ and δ genes. This process gives rise to the αβ and γδ T cell lineages (132). Finally, thymocytes that are committed to the αβ lineage must differentiate along either the $CD4^+$ or $CD8^+$ mature T cell lineages or a small population (about 5% of total thymocytes) of $CD4^-CD8^-$ double negative (DN). Subsets of this αβ DN T cell population are $NK1.1^+$ T cells (133) and $TCR\alpha\beta^+CD3^+Thy1^+$, which some cells lack expression of NK1.1 and TCR γδ markers (134, 135).

2.2.1. Effector T cells

- *$CD4^+$ T cells and $CD8^+$ T cells*

It has been well established that peptide presentation to αβ T cells requires the engagement of two sets of receptors: CD3: αβTCR: MHC-I/MHC-II and co-stimulator, and the activation of both CD4+ and CD8+ T cells is necessary for optimum immunity against tuberculosis (136). CD4+ and CD8+ T cells possess cytotoxic activities and produce essential macrophage-activating cytokines, such as IFN-γ and TNF. IFN-γ produced by M.tb-specific T cells, is an important source in activating macrophages which leads to the antibacterial activity. Genetic disruption of IFN-γ in mice resulted in disseminated tuberculosis (137). TNF-α/β can synergize with IFN-γ in macrophage

activation, and in killing some target cells through their interaction with TNF receptor-1. Neutralisation of TNF-α by a monoclonal antibody causes reactivation of latent infection in mice and humans (138, 139). CD4+ T cells are important for the initial control of the bacterial growth as well as for reactivation of the disease (140), whilst CD8+ T cells are important for the control of later stages of infection (141). Depletion of CD4+ T cells causes disease reactivation in chronically infected mice (107) and a growing body of evidence suggests that depletion of CD4+ cells or impairment of their function are major consequences of HIV/TB co-infection, leading to reactivation and progression (5, 18).

In addition to cytokine production, both CD4 and CD8+ T cells mediate cellular cytotoxicity, though this is a less significant property of CD4+ T cells. Mechanisms of T cell-mediated cytotoxicity are: the release of cytotoxic granules containing perforin and granzymes, CD95/CD95L (Fas/Fas Ligand)-dependent killing, and the production of TNF-α that induces apoptosis in some sensitive targets (142-144). The granule-mediated killing is strictly calcium-dependent and is the main way by which cytotoxic CD8 T cells eliminate infection. CD8+ cytotoxic T lymphocytes (CTLs) were found to be present in the lungs and lung-draining lymph nodes of mice infected with *M. tuberculosis*, with the ability to recognize and lyse M.tb-infected macrophages in a perforin-dependent manner (143). Lysis of *M. tuberculosis*–infected macrophages could be beneficial if the released bacteria are taken up by activated macrophages that can mediate bacterial killing. Conflicting results were reported in the timing of M.tb-specific CD8+ cytotoxicity in infected mice, though both studies concluded that there is an important role of CD8+ T cells in the long-term control of M.tb infection (141, 142).

- *CD1-restricted T cells*

The cluster of differentiation 1 (CD1) gene is conserved in almost all mammals and functions as alternative antigen presentation pathway to T cells (145). The human CD1 family (CD1a, b, c, d and e) are heterodimers and map outside the MHC (146). Based on the structural similarity in the extracellular domains of α1 and α2, the human CD1 family can be divided into two groups, group 1 (CD1a, b and c) and group 2 (CD1d) (147). CD1e is regarded as a separate group due to its unique structural characteristics and cellular localisation (148). Group 1 CD1 molecules are predominantly expressed on professional APCs (e.g. DCs, macrophages and B cells), whilst group 2 CD1d is mainly expressed on B cells and thymocytes and on intestinal epithelia (147, 149). Unlike conventional αβ T cells, which are selected by classical MHC class I and II, some T cells are selected by the CD1 molecule, which are termed 'CD1-restricted T cells' (150). Another characteristic of CD1-restricted T cells is the diverse expression of co-receptors. They can be CD4+ or CD8+ or $CD4^{-}CD8^{-}$ double negative (DN) T cells as well as γδ T cells (147, 151). CD1d mainly presents lipids to invariant natural killer cells (iNK T cells), which express NK markers as well as a single invariant Vα14 T cell receptor. However, small subsets of NK T cells are either 'CD1d-dependent non-Vα14 NKT cells', which express diverse receptors and constitute a minor population expressing $CD8^{+}$, or CD1d-independent, which are the conventional CD4+/CD8+ T cells that co-express NK markers (e.g. NK1.1, DX5) (152).

An unusual feature of CD1 protein is that it presents glycolipid antigens rather than peptides, although recent views suggest a much broader antigen diversity including lipopeptides and endogenous peptides (148, 153). Although Group 1 CD1 molecules are absent in rodents, they are present in human and several M.tb cell wall components

are reported to be recognised by group 1 CD1 (148). Mycobacteria can be internalised by different types of PRRs expressed by APCs (e.g DC-SIGN, mannose receptor) and breakdown within acidified endosomal compartment, where the CD1 molecules are loaded. CD1-restricted T cells were shown to respond to several mycobacterial components (145, 154) and to initiate IFN-γ production independently of MHC class II (151). A small subset of DN T cells is TCR$\alpha\beta^{+}CD3^{+}Thy1^{+}$, and some cells lack expression of NK1.1 and TCR γδ markers, and are important in controlling growth of the intracellular bacterium *Francisella tularensis* live vaccine strain (LVS) (134). A further report showed that DN T cells are potent effector cells that expand after infection with *F. tularensis* and M. tb and change from effector to memory phenotype after the contraction phase of the T cell response (135).

An investigation into the cytotoxicity of CD1-restricted T cells (CD4-CD8- double negative and CD8+ αβ T cells) against M.tb-infected macrophages showed phenotypically-dependent intracellular killing (155). The cytotoxicity of CD4-CD8- DN T cells was mediated by Fas-FasL interaction, whilst the CD8+T cells lysed infected macrophages by a granule-dependent mechanism that resulted in killing of bacteria. A study that examined *ex vivo* T-cell responses to natural lipid antigens of M.tb in latently infected individuals found that lipid antigen triggers the recall response to *M. tuberculosis*, suggesting that CD1-restricted T cells are present in the circulating blood pool (156). Apart from initiation of cell-mediated immunity, NK T cells can also rapidly induce humoral and Th2 responses, such as IgE and IL-4 (133). However, humoral and Th2 cytokine levels were not significantly altered in mice defective in CD1 (157, 158).

- *γδ T cells*

T cells bearing γδ receptors differ from T cells in the type of antigen they recognise and the CD4 and CD8 co-receptors expressed on the surface, but function similarly to αβT cells in terms of subsequent cellular response, cytotoxic activity and memory cell generation after the first encounter with antigen (159). Unlike the situation in the mouse, there are two major subsets of γδ T cell in humans. Vγ2Vδ2 T cells (also known as Vγ9Vδ2) predominate in the circulation and Vγ2Vδ1-encoded TCRs account for the vast majority of γδ T cells in tissues such as intestine and spleen. In normal human peripheral blood, γδ T cells constitute 2 to 10% of the total T cell pool, with the majority (60-95%) expressing the Vγ2Vδ2 TCR (160). γδ T cells have long been considered as innate-like immune cells because of their rapid innate-like immune response which leads to direct kill of microbial invaders (161). Recently, Brandes and colleagues have demonstrated that activated Vγ2Vδ2 T cells, by up-regulating cell surface expression of costimulatory and MHC II, can efficiently process and display peptide antigen to CD4+ αβT cells, exerting professional APCs function similar to that of DCs (162). Therefore, γδ T cells may act as a bridge between innate and adaptive immunity. However, how antigens are processed as well as their nature remains unidentified. Some reports suggested antigen recognition in the context of CD1c-Vδ1-expressing γδ T cells (163) whereas Vγ2Vδ2 T cells recognize antigen directly by TCR diversity (164, 165). Human $V\delta2^+$ T cells recognize mycobacterial phospholigands conserved in metabolic pathways (e.g. phosphoantigens, alkylamines) and synthetic aminobisphosphonates used in cancer therapy, whereas $V\delta1^+$ T cells recognize mycobacterial glycolipid presented by CD1c (163, 166).

Several lines of evidence indicate that γδ T cells play an important role in immunity against tuberculosis with both characteristics of innate and adaptive-like immune

responses (167, 168). The first studies suggesting a link between γδ T cells and mycobacteria were conducted by Janis *et al.* reporting an increase in γδ T cells in draining lymph nodes of B10A mice immunized with complete Freund's adjuvant (169). Barnes *et al.* established that patients with pulmonary tuberculosis had a diminished ability to expand γδ T cells *in vitro* in response to heat-killed M.tb and IL-2 (170). Studies at a chronic phase of tuberculosis showed a decrease in number of $V\gamma2V\delta2^+$ T cells in both the blood and BAL fluid of patients with pulmonary tuberculosis when compared with healthy controls (171) and this decreasing in γδ T cells resulted in reduced IFN-γ production (172). Cytotoxicity mediated by γδ T cells against targets pulsed with M.tb was dependent upon activation through the TCR (173). An i*n vitro* study comparing the $CD4^+$ and γδ T response against M.tb infection in macrophages suggested that $CD4^+$ and γδ T cells have similar effector functions in terms of cytotoxicity and IFN-γ production (174). Interestingly, within the effector memory subset, Vδ2 T cells can be functionally grouped into IFNγ/TNFα-producing effectors and cytotoxic effectors, based on expression of CD16 (FcgammaR-III) (175).

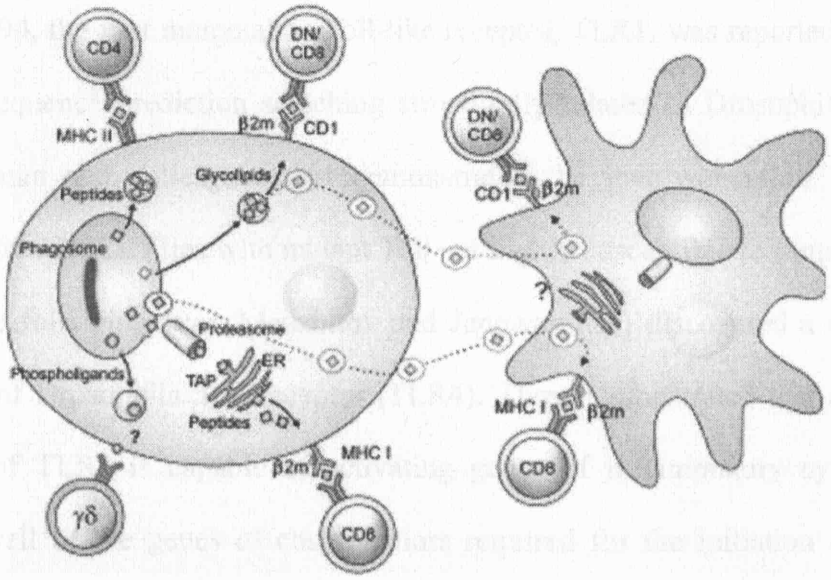
2.3. Tuberculous granulomas

Inhaled droplets containing mycobacteria are engulfed by alveolar macrophages. Activated macrophages then recruit effector T cells to the site of infection, resulting in a small granulomatous lesion containing the bacillus in the lung. *Mtb* is able to persist in the early phagosomal compartment even when acquired T cell immunity has been established, by inhibiting phagosome maturation and phagolysosome fusion (176). Although T cells are the major mediators of protection, immediate effector functions

are mostly provided by macrophages. Therefore, the cross talk between macrophages, DCs and effector T cells (e.g. CD4 T cells, CD8 T cells, γδT cells, and CD1 restricted T cells) is the key to maintaining the homeostasis of the immune balance in the granulomas. The cross talk between T cells and macrophages is orchestrated by various cytokines, such as IFN-γ, TNF-α, and IL-12. IFN-γ synergises with tumour necrosis factor α (TNF-α) in activating macrophages, resulting in direct killing or control of mycobacterial replication (9). IL-12 functions in polarising Th1 cells (177). TNF is a key cytokine for granuloma formation because neutralization of TNF results in reactivation of tuberculosis-infected mice (1). This observation was confirmed in humans with rheumatoid arthritis treated with anti-TNF-α antibodies (178).

Secondly, this cross talk causes the formation of well organised granulomas, where professional APCs (e.g. macrophages and DCs) and different T cell populations exist. Because the initial inflammatory response induced by the bacterium is crucial to the formation of the granuloma, how mycobacterial products influence the inflammatory response has been intensively studied. As discussed above, each type of effector T cell recognises different mycobacterial products by various antigen presentation pathways, hence acting as central mediators of protection. In summary, CD4 T cells recognise antigenic peptides in the context of gene products encoded by the major histocompatibility complex (MHC) class II and CD8 T cells recognise antigenic peptides in the context of MHC class I. Phospholigands, metabolic products of mycobacteria, stimulate γδT cells in the absence of known antigen presentation molecules. CD1 restricted T cells recognise glycolipids abundant in the mycobacterial cell walls presented by CD1 molecules. Presentation of proteins by MHC class I and of glycolipids by CD1 is more complex and probably requires cross priming (179). Mycobacteria-infected macrophages can undergo apoptosis and provide access to

antigens for bystander dendritic cells. Uptake of these apoptotic vesicles results in glycolipid presentation through CD1 and protein presentation through MHC class I in DCs. This two cell mechanism can explain stimulation of MHC class I restricted CD8 T cells and of CD1 restricted T cells.



Adapted from Kaufmann, S H E Ann Rheum Dis 2002;61 :54ii-58ii
(Reproduced from Nature Reviews Immunology (vol 1:20-30).

Figure.1.3. Antigen cross-presentation pathways in tuberculosis granulomas. Mycobacterial antigens reside in the early phagosome in macrophages, where bacterial proteins are presenting to CD4 T cells by MHC class II. Phospholigands, metabolic products of M.tb, stimulate γδT cells in the absence of known antigen presentation molecules. Presentation of proteins by MHC class I and of glycolipids by CD1 is more complex and may require cross priming between macrophage and DCs.

3. TOLL-LIKE RECEPTOR

3.1. History of TLR

The research history of Toll-like receptors (TLRs) is relatively short but is constantly being updated. Toll was first identified in Drosophila by Hashimoto et al in 1988 as the gene required for the development of the dorso-ventral pattern in developing embryos (180). In 1994, the first mammalian Toll-like receptor, TLR1, was reported by the use of coding sequence prediction searching structurally related to Drosophila (181). In 1996, Hoffman and colleagues first demonstrated the involvement of Toll in the immune system (182). Flies with mutant Toll are highly susceptible to fungal infection (182). In the following year, Medzhitov and Janeway (20) discovered a mammalian homologue of Drosophila Toll receptor (TLR4). They demonstrated that constitutive expression of TLR4 is capable of activating genes of inflammatory cytokines via NF-kB as well as the genes of costimulators required for the initiation of adaptive immunity. A recent study suggested that this up-regulation of costimulatory molecules such as CD80 and CD86 is dependent upon adapter proteins of TLR and upon type I interferon receptor signalling (183). The work of Medzhitov and Janeway was a milestone in the history of immunology by addressing the question of how innate immunity is linked to adaptive immunity through the activation of PRRs. Sequencing of the Drosophila genome revealed the existence of nine proteins belonging to the Toll families (184). 11 families of human TLR have subsequently been identified (21).

3.2. Biology of TLR

3.2.1. TLR protein structure

TLR have an extracellular domain with a variable number of N-terminal leucine rich repeat motifs (LRRs) followed by a cysteine region, a transmembrane (TM) domain, and an intracellular toll/interleukin-1 receptor (TIR) domain. Due to a considerable homology in the cytoplasmic region, TLR are members of the large IL-1R/TLR superfamily, which consists of three subgroups (185). Members of the first group constitute immunoglobin domains in the extracellular regions, such as IL1, IL18 receptors. The second group of members contain extracellular LRR motifs, such as the TLR family and the third group includes intracellular adaptor proteins that are necessary for signalling, such as MYD88 (186). Although the extracellular region of the TLRs and IL-1Rs differs significantly, both receptors share a weakly conserved intracellular TIR domain, with a 26% identity and a 43% similarity in amino acid sequence (187). The variation in amino acid number (150-200) and molecular weight of the TLR family is due to the differences in the number of extracellular LRRs, while the cytoplasmic TIR domain is shared by the family (188). Although there is a 50% amino acid sequence identity of TIR domain between TLR1 and TLR2, authors indicated clear structural differences between them (188).

In general, the extracellular LRR domain is important for ligand recognition whilst the TIR domain is required for the initiation of signal transduction. Both domains are important in protective immunity and mutations in either domain resulted in susceptibility to infection (189, 190). For example, a single-site mutation (Pro712His) in the TIR domain of TLR4 renders mice unresponsive to LPS (189) and a mutation in the intracellular domain of TLR2 (Arg677Trp), has been linked with lepromatous

leprosy in Korea (27). Cells transfected with a deletion mutant in the TLR2 extracellular residues Ser^{40}-Ile^{64} failed to respond to *S. aureus* peptidoglycans (PGN) (190). Subsequent studies showed that a leucine-rich repeat motif was also involved in the recognition of mycoplasmal diacylated lipoproteins, lipopeptides and *S. aureus* PGN, suggesting that peptides may be recognized by more than one LRR motif (191). Though Meng and colleagues (192) argued the recognition of synthetic diacyl and triacyl- peptide is by a region including the N-terminal seven LRR/LRR-like motifs rather than by single LRRs, both studies concluded that TLR2 contains multiple binding domains for ligand recognition (191, 192).

3.2.2. Phylogenetic tree of human TLR

Based on the amino acid (or genomic) sequence similarity, the members of TLR can be grouped into 5 subfamilies: TLR2, TLR3, TLR4, TLR5 and TLR9 families (193). The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10; the TLR9 subfamily is composed of TLR7, TLR8, and TLR9. In the TLR2 subfamily, TLR1 and TLR6 share a high similarity in genomic structure (one CDS, located in same chromosome, 4p14) as well as protein structure, exhibiting 69.3% identity in overall amino acid sequence and with over 90% identity in the TIR domain (194). It has been proposed that TLR1 and TLR6 may be the products of an evolutionary duplication. Of note, TLR3 has a unique structure among the TLRs in that it has five exons and the protein is encoded by exons 2 through 5. This is different to the other members, which are encoded by only one or two exons. Members of the TLR9 subfamily, TLR7, TLR8, and TLR9, are encoded by two exons (195). The genes for TLR7 and TLR8 show 42.3% identity and 72.7% similarity in their amino acid sequences, have similar genomic structures, and are located close to each other on the X chromosome (195).

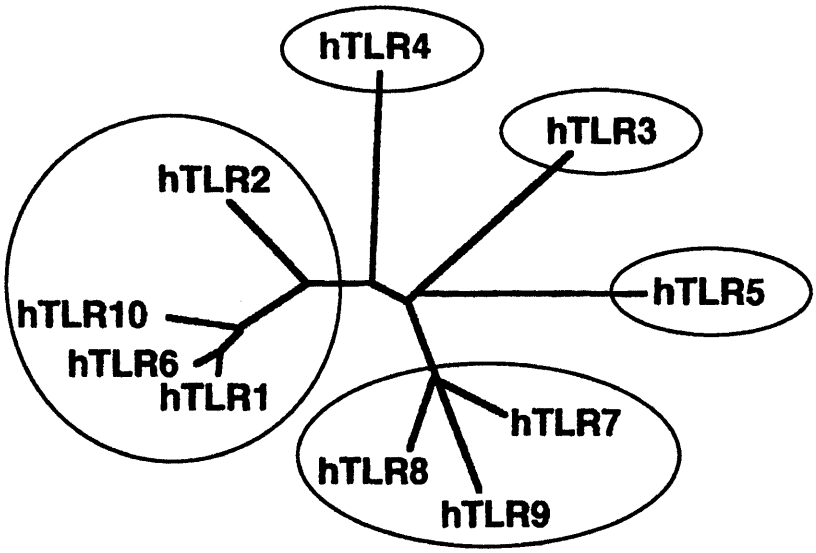


Figure 1.4. The phylogenetic tree of the TLR family. Based on the amino acid (or genomic) sequence similarity, the members of TLR can be grouped into 5 subfamilies: TLR2, TLR3, TLR4, TLR5 and TLR9 families. The phylogenetic tree was derived from an alignment of the amino acid sequences for the human TLR members using the neighbour-joining method (Adapted from Takeda K et al, 2003, Annul. Rev. Immuno, v21, 335-376) (193).

3.2.3. Signalling pathways

Until recently it was unclear how the infected macrophages are able to link the innate immune response to adaptive immunity and the discovery of TLR addresses this question (25). It has been established that ligation through TLRs triggers at least two important signals: 1) A myeloid differentiation factor (MyD88)-dependent pathway, (one of the adaptor proteins shared by all the TLRs), leads to the activation of the transcription factor NF-kB, which governs the release of proinflamantory cytokines required to control the infection. 2) A second signal, which is MyD88-dependent/independent, drives maturation of APCs and increases expression of markers such as MHC class, costimulators, CD40 and the chemokine receptor of CCR 7 for migration to the nearby draining lymphoid node. MyD88 is a 35kDa intracellular protein that contains three functional domains: a death domain, an intermediate domain and a TIR domain. MyD88 is a critical protein for signalling by IL1-RI, IL-18R and all 11 members of TLR family, where it is recruited to the receptor complex and acts as adaptor protein to interact with downstream signalling molecules once activated.

So far, a total of four TIR domain containing adaptors: MyD88, MAL (MyD88 adaptor like protein)/ TIRAP (TIR domain-containing adaptor protein) (196) (196, 197), TRIF (TIR domain-containing adaptor inducing IFN-β) (198), TRAM (TRIF-related adaptor molecules) (199) have been shown to have the ability to interact directly with the TIR domain of TLRs. Both TRIF and TRAM produce type I interferons (IFNs) independent of MyD88 in response to antigen stimulation via TLR3 and TLR4 respectively (198, 199). How activation of specific TLRs and adaptor proteins leads to different patterns of gene expression is being extensively studied. The activation of TLR3 and TLR4 leads to induction of IFNs, but activation of TLR2 and TLR5 does

not (198, 199). It is now well documented that a MyD88-independent pathway exists leading to the production of IFNs in addition to MyD88-dependent pathway. These findings support the idea that there is more than one TLR-signalling pathway that controls cytokine gene expression.

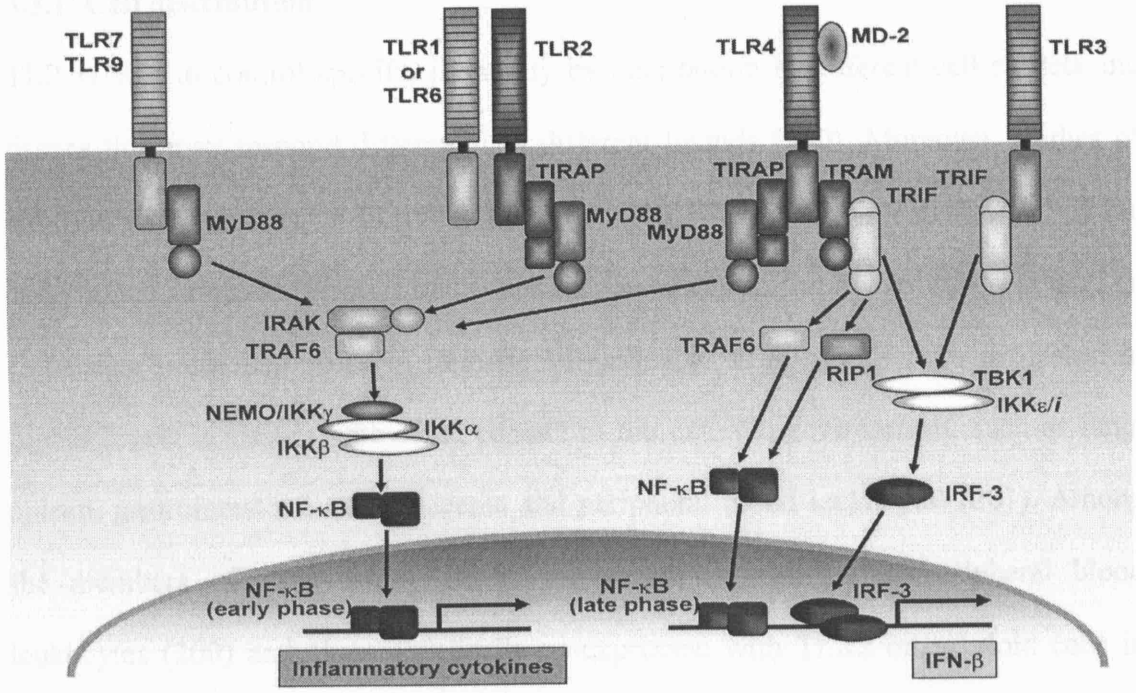


Figure 1.5. TLR signaling pathway (Adapted from Takeda K and Akira S, 2005, Int Immunol, v17, no1) (21). Upon stimulation, MyD88 recruits IRAK-4 to TLRs through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6, leading to the activation of two distinct signaling pathways: MAP kinases and transcription factor NF-κB. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. Both TRIF (TLR3) and TRAM (TLR4) produce type I interferons (IFNs) independent of MyD88 in response to antigen stimulation. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

3.3. Immuno-characteristics of TLR

3.3.1. Cell distribution

TLR is able to control specific immunity by distribution in different cell subsets and tissues that may respond differently to different ligands (200). Moreover, studies of subcellular localisation of TLRs indicated that TLR1,2,4,5 and 6 are distributed on the cell surface, whereas TLR3,7 and 9 are expressed intracellularly in endosomes (21). Consistent with their roles in immune surveillance, TLR mRNAs are expressed at higher levels in tissues with close contact to the external environment, such as lung, spleen, gastrointestinal tract, placenta and peripheral blood leukocytes (201). Among the members of TLR family, TLR1 expression is high in all peripheral blood leukocytes (200) and characteristically co-expressed with TLR2 on myeloid cells in lymphoid tissue examined by immunohistochemical techniques (202). Myeloid cells, in particular monocytes, macrophages and dendritic cells, are the dominate cell type expressing TLR1, TLR2, TLR4 and TLR6 whereas TLR3,7,9 and TLR10 are restricted in DCs and B cells, respectively (200, 201, 203).

Differential expression of TLR by myeloid dendritic cells (mDCs) and plasmacytoid DC (pDCs) has been suggested to influence the type of immune response (203). A study of localisation of TLR3 in human DC subsets revealed that myeloid-lineage DCs express TLR3 in their cytoplasmic compartment whilst BDCA-4^{+} plasmacytoid DCs do not express TLR3 either on the cell surface or in cytoplasm (204). In contrast to myeloid DCs, human pDCs selectively express TLR7 and TLR9 within the endosomal compartment and the activation of pDC via these TLR triggers the release of type I IFN (alpha/beta/omega) through the MyD88-dependent pathway, which is important in eliminating virus infection (205). Notably, TLR3 expressed by myeloid DCs acts via the TRIF adaptor protein, MyD88-independent pathway, to trigger type I IFN secretion

(198). In the light of the immunoregulatory role of TLR, Caramalho et al reported that $CD25^+$ $CD4^+$ naturally occurring regulatory T cells in normal naïve mice selectively express TLR4, 5, 7 and TLR8 (TLR1, 2 and TLR6 are expressed on all CD4 cells) (206). Stimulation of $CD25^+$ $CD4^+$ cells with lipopolysaccharide (LPS), a TLR-4 ligand, induces activation and enhances their survival/proliferation in the absence of APCs, indicating that LPS directly acts on TLR4 receptor expressed by this type of regulatory T cell (Treg).

3.3.2. Ligand specificity of TLR

TLRs recognise conserved pathogen associated-molecular patterns (PAMPs) present on a wide range of different microbes (20). In general, Gram-positive bacteria and mycobacterial cell wall components are mainly recognised by TLR2, whilst Gram-negative bacteria are recognised by TLR4 (22). TLR2 can form heterodimers with TLR1 or TLR6 to distinguish subtle differences between diacyl and triacyl lipopeptides (23, 24). TLR4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen (21), although it is claimed that the activation of HSP-TLR4 is due to the contamination of LPS (207). Double-stranded RNA (dsRNA) is the ligand for TLR3 (208) and bacterial flagellin is recognized by TLR5 (209). TLR7 and TLR8 are structurally related proteins and recognise synthetic compounds, imidazoquinolines, which are structurally related to guanosine nucleoside and is clinically used for treatment of genital warts associated with viral infection (210). This notion led to the identification of a nucleic acid-like structure of viruses: guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus,

vesicular stomatitis virus and influenza virus (211-213). TLR9 is essential for recognition of CpG DNA (214), which as it is unmethylated, is recognised as bacterial. Taken together, TLRs appear to recognise a diversity of ligands, ranging from conserved motif s of pathogens, to synthetic peptides and endogenous ligands.

3.4. The major groups of bacteria and TLR2 agonists

3.4.1. Classification of bacteria species

Some major groups of bacteria have traditionally been distinguished by their staining properties: The Gram stain uses crystal violet and iodine to distinguish between Gram negative (-) and Gram positive (+) bacteria. The mycobacteria are characterized by positive Ziehl Neelsen staining. These staining reactions are properties of the cell wall, and are also related to each group's distinct immunostimulatory properties. Mycobacteria are resistant to penetration by many antimicrobial agents or Gram staining due to a layer of mycolic acids and other lipids which render the bacterial cell surface waxy and highly hydrophobic. The mycobacterial cell wall contains a high proportion of lipids and lipoproteins. Lipoarabinomannan (LAM) is exposed on the cell surface, but also passes through the lipid and peptidoglycan layers, and its lipid components are inserted into the cell membrane. LAM, lipid/lipoproteins and PGN are important components for pathogenesis (215). The Gram (+) bacterial cell wall contains a thick layer of peptidoglycan (PGN) and also lipoteichoic acid (LTA). The LTA passes through the peptidoglycan layer and is inserted into the inner cell membrane. In contrast, the Gram (-) bacterial cell wall has relatively thinner peptidoglycan layer but it contains a unique outer membrane (lipid bilayer) into which is inserted lipopolysaccharide (LPS). The detailed structure of LPS is strain-dependent. The biologically active component of LPS is the glycolipid (Lipid A), though other

modifications (lipid chain lengths and carbohydrate composition) modify its properties. Most of the mycobacteria are harmless species except for *M. tuberculosis* and *M. leprae* which can cause chronic infections in humans. Other species such as members of the *M. avium/intracellulare complex, M. scrofulaceum, M. kanasii* and various members of the fast growing subgroup of mycobacteria, cause opportunistic infections.

3.4.2. Diversity of TLR2 agonists

TLR2 recognises a broad spectrum of microbial components including lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram(+) bacteria, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum* (193). Moreover, purified LPS from *H. pylori* strain 26695 (216) and *Leptospira interrogans* (217) activated NF-kappa B in cells via TLR2 but not via TLR4.

3.4.3. Ligands of TLR2/TLR1 and TLR2/TLR6 heterodimers

Lipoproteins and lipopeptides are the most extensively studied components due to their presence in a variety of pathogens, such as Gram (-)/(+) bacteria, mycobacteria and also mycoplasma species. The immunostimulatory component of lipoproteins is located in the region of the N-terminal acylated lipopeptide and bacteria may differ in the degree of acylation in their N-terminal residues. Takeuchi and colleagues (23) first tested the hypothesis that TLR2 might distinguish different lipid configurations by

pairing with either TLR1 or TLR6. By generating TLR6 KO mice, they examined TLR $6^{-/-}$ macrophage's response to a synthetic mycoplasmal lipopeptide (MALP-2), which is diacylated, and a synthetic triacylated bacterial lipopeptide (PAM_3CSK_4). They demonstrated that the TLR1/2 heterodimer binds triacylated lipopeptides, whereas the TLR2/6 combination is specific for diacylated lipopeptides. Subsequent studies by other groups confirmed that most TLR2 ligands, mainly lipoproteins and lipopeptides, are recognised by heterodimers of TLR2 and it's co-receptors TLR1 or TLR6, and possibly other members of the TLR family (43, 218).

In summary, TLR2/TLR6 heterodimers recognise the lipoteichoic acid (LTA) of Gram-positive bacteria, and the peptidoglycan (PGN), macrophage-activating lipopeptide-2 KD (MALP2) which is derived from mycoplasma, zymosan (yeast cell wall particle) and Soluble Tuberculosis Factors of Mtb (23, 219-221). Agonists for TLR2/TLR1 include the 19kD lipoprotein of mycobacterial, the synthetic lipopeptide Pam3CysK4, ara-lipoarabinomannan (araLAM) of mycobacteria, the OspA antigen of *Borrelia burgdorferi* and soluble factors of *Neisseria meningitidis* (24, 43, 218, 222). Mycobacterial lipoarabinomannan (LAM) is also a ligand for the CD14/TLR2 receptor complex (223) as well as for the recently identified lectin DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) (35, 125). The 24 kDa LprG of Mtb is a ligand for TLR 2, although it is not currently known whether TLR1or TLR6 is also involved (36).

3.4.4. Mechanisms of diverse ligand recognition by TLR2

- Association of TLR2 with TLR1 and TLR6

Heterodimerisation of TLR1 or TLR6 accounts for the diversity of ligand recognition by TLR2 (224), though the underlying recognition mechanisms as well as the structural details of the ligand binding site remain unclear (191, 192). TLR1 and TLR6 are the known partners of TLR2 with no independent functional capacity. Both receptors are structurally related to TLR2 and both share 69% identity in their amino acid sequence (194). Evidence from studies of chimeras, either with constitutive or transient expression, showed that TLR2 physically associates with its partner in a ligand-independent manner (218, 225). This association was mediated by the extracellular domains and deleting the extracellular domain abolished this association, but not cytoplasmic domains (225).

Although the ligand binding site for TLR1 and TLR6 remains to be elucidated, it has been reported that TLR1 is essential for recognition OspA, an outer surface lipoprotein of *Borrelia burgdorferi* (226). Macrophages from TLR6-deficient mice did not show any production of inflammatory cytokines in response to mycoplasma-derived diacyl lipopeptides but they retained responsiveness to triacyl lipopeptides derived from Gram-negative bacteria (23). Blocking TLR6 surface receptor by mAB (TLR6.127) inhibited cytokine production in monocytes and immature dendritic cells (iDCs) treated with MALP-2 or peptidoglycan but not with Pam3CSK4 and LPS, suggesting that TLR6 recognized it's ligands at the cell surface (227). Similar results were obtained in experiments that involved blocking human TLR1 and TLR2 or using cells derived from TLR1-defective mice (24, 218). Anti-TLR1 (GD2.F4) and anti-TLR2 (11G7 clone) mABs block IL-6 production in PBMCs stimulated with the TLR1/TLR2 agonists AraLAM and Pam3CysK4 but not Zymosan, which is an agonist of

TLR2/TLR6 (218). Those results indicate that TLR1 and TLR6 exert influence on TLR2 ligand-specificity as well as on subsequent cellular activation. Since anti-TLR1 or anti-TLR6 exhibit similar inhibitory effects on cytokine production as anti-TLR2, it is tempting to speculate that the binding sites for different ligands are formed by the dimerisation of TLR2 to TLR1/TLR6. This is supported by the evidence that TLR2 physically associates with TLR1 or TLR6 in their extracellular domains (191).

- Association of TLR2 with other PRRs

Wang *et al* observed that LPS binds to CD14 in low-density domains of the monocyte-macrophage (THP1) plasma membrane and proposed that associations of pattern recognition receptors in the plasma membrane facilitate antigen discrimination (228). Subsequently, `Lipid rafts' were proposed as lateral structural components of the plasma membrane which function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction (229). TLR4 was found to be recruited in membrane microdomains (lipid rafts) following stimulation by LPS and subsequent lipid raft integrity was crucial for LPS-induced cellular activation. (230). Recently, the same group further demonstrated that addition of lipid raft disrupting drugs (methyl-β-cyclodextrin) prior to stimulation by bacterial products abolished the confinement of TLR2 and TLR4 within lipid rafts and resulted in no formation of immobile clusters and LTA-induced TNF-α secretion was dramatically inhibited (231). Other reports also revealed that TLR2 cooperates with other pattern recognition receptors: with scavenger receptors in facilitating p38-dependent phagocytosis and with dectin-1, a lectin family receptor, for the recognition of the fungal cell wall component β-glucan (232, 233).

Taken together, the innate immune system may use cooperation between toll-like

receptors (224) or combinational associations of TLRs with other PRRs (232) to discriminate a broad spectrum of mycobacterial antigens. Also, TLR may not act as the main receptors for ligand recognition but rather as the integrators for the downstream signalling response. The activation mechanism used by innate immune cells in response to pathogens requires further investigation.

3.5. Evidence of host defence against mycobacterial infection by TLR

3.5.1 TLRs and anti-mycobacterial mediators

iNOS and NO expression are induced by a wide variety of inflammatory mediators such as TNF-α, IFN-γ, IL-1β and mycobacterial components (234). TLR signal transduction leads to the expression of several proteins that have important roles in the innate immune response to pathogens, such as antimicrobial proteins and pro-inflammatory cytokines. Adams and co-workers (235) showed that the LAM derived from the virulent Erdman strain of *M. tuberculosis*, in conjunction with IFN-γ, induced NO expression in mouse peritoneal macrophages. Chan and colleagues reported that *M. tuberculosis* LAM alone is capable of inducing iNOS-NO expression in a mouse macrophage cell line RAW 264.7 that is poorly responsive to TNF-α or IL-1β (236). Ligand characterisation studies suggested that LAM and its precursors, phosphatidylinositol mannosides, are recognised by heterodimers of TLR1 and TLR2 and recognition of antigen leads to the initiation of NF-kappaB-driven luciferase activity (237). Using a TLR2 dominant negative mutant model in the murine RAW 264.7 macrophage cell line, Brightbill and colleagues demonstrated that the 19-kDa lipoprotein of *M. tuberculosis* induced iNOS promoter activity via TLR2 (238). This led to a further investigation of the involvement of TLR-induced NO production in the

initiation of antimicrobial activity after cellular activation. It was shown that TLR2 activation led to killing of intracellular *M. tuberculosis* in both mouse and human macrophages by distinct mechanisms (239). In mouse macrophages, activation of TLR2 by bacterial lipoprotein leads to nitric oxide-dependent killing of intracellular tubercle bacilli, but in human monocytes and alveolar macrophages, this pathway was nitric oxide-independent.

Genetically defective mice showed impaired levels of iNOS or NO in TLR2/TLR4-/- mice (30, 32, 240, 241). A study of the signalling pathway using MYD88-/- infected mice revealed that both MYD88-dependent and MYD88-independent pathways are required for the anti-mycobacterial activity (242, 243). Studies led by Fenton *et al* found that, contradictory to LPS-TLR4, TLR2 agonists (LAM, STF, PIM) failed to produce NO in both human alveolar macrophages and murine RAW 264.7 but treatment of IFN-γ confers on TLR2 agonists the ability to induce NO production. (220, 244). Authors suggested that type I IFNs (α/β) may act as a missing signal in the elicitation of NO by TLR2 agonists. This observation is supported by a recent study which showed the suppression of LPS-induced iNOS expression was correlated with the downregulation of IFN-β expression (a MyD88-independent gene) and subsequent decrease in STAT1 phosphorylation by the inhibitors of Src-family tyrosine kinases (STKs), PP1 and SU6656, in MyD88-knockout as well as wild-type macrophages (245). Therefore, STKs may play a positive regulatory role in TLR4-mediated iNOS expression in a MyD88-independent (TRIF-dependent) manner.

3.5.2. Evidence from knockout mouse studies

The important role of TLR 2 and TLR4 as the centre of host defense against microbial infections was demonstrated in a human genetic study (27, 28), though it remains controversial in studies of mice with genetic defect in TLR2 and TLR4 (30, 32, 241, 246). Knockout mouse studies demonstrated that TLR 2 is important in controlling (30) or surviving airborne infection with *M. tuberculosis* (32), depending on the concentration of infecting organisms. The standard low-dose model of infection (100 CFU/mouse) showed that both wild type and TLR2 KO mice survived the duration of infection, though the TLR 2 KO mice developed larger granulomatous pulmonary lesions in lung, whereas levels of antimicrobial mediators and cytokines in lung tissues, such as inducible nitric oxide synthase (iNOS) and TNF-α were lower than in wild-type mice (30, 32). Subsequent challenge with a higher dose (500 CFU/mouse) resulted in early death in TLR2 KO mice (32). Conflicting data exist describing the role of TLR4 in controlling M. tuberculosis infection in mice (29, 221, 241, 246). Although the KO models provide an extremely powerful tool to understand the functional role of a specific gene in a real physiological situation, one could argue that genes may compensate for one another after one has been deleted. Moreover, different species may use different regulation to control specific genes (247).

3.5.3. Evidence from human genetic studies

Polymorphisms of TLR2 and TLR4 have been linked to the susceptibility to allergy (248, 249) and leprosy (27, 28, 31, 250). *Mycobacterium leprae* and *Mycobacteium tuberculosis,* are genetically closely related, and are the known intracellular pathogenic strains that cause chronic infection in human. Once infected with *M. leprae*, like Mtb, the outcome of the disease mainly depends on the host immunity, in particular

cell-immediate immunity. Unlike Mtb, infected patients may develop two types of leprosy: tuberculoid leprosy (TT) which is characterized by Th1 cytokines in the lesion, and lepromatous leprosy (LL) which has a Th2-like profile. Kang *et al* first reported that ta mutation in the intracellular domain of TLR 2 Arg677Trp is associated with lepromatous leprosy in a Korean population (27). The TLR 2 mutation was found in 22% of LL patients but not in the patients who developed the tuberculoid form (TT). A subsequent study showed that the TLR 2 Arg677Trp mutation abolished the cellular response to both *M. tuberculosis* and *M. leprae* (250) because TLR 2 and TLR 1 are the main receptors to recognize and initiate effective immunity in response to heat killed *M. leprae* and *M. leprae*-derived 19-kD/33-kD lipoproteins (43). The TIR domain of TLRs is shared by the family and is involved in signal transduction. Mutation in this conserved region can abolish the cellular response. This may explain partly why mutation in TLR 2 is associated with patients with LL leprosy in which they are more susceptible to the pathogen and have Th2 like cytokines in their lesion. Polymorphisms in other positions of human TLR such as TLR 2Arg753Gln (31) and TLR 4 at residues 299 and 399 (28) have also been reported to result in disease susceptibility. Notably, low expression of TLR1, was reported in association with hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in a study in humans and mice (226). Therefore it is likely that a defect in either TLR 2 or a co-receptor of TLR2 (TLR 1 or TLR 6) or TLR4 can increase the risk of infection.

3.6. Immune invasion mechanisms used by mycobacteria

Macrophages utilize phagocytic receptors as well as other PRRs to recognise and phagocytose the bacilli. Organisms that cause chronic infection must be able to subvert defense mechanisms orchestrated by macrophages. Indeed, intracellular mycobacteria have long been reported to inhibit phagosome maturation (101). This is important for intracellular bacterial survival since maturation of the phagosome triggers release of anti-microbial mediators e.g. reactive oxygen and nitrogen compounds, which are important for bacterial killing. It is beginning to emerge that in addition to their role in triggering protective immunity, TLR are also targeted by mycobacteria as a means of immune evasion (34). Firstly, mycobateria inhibit phagosome maturation by targeting the TLR pathway. Underhill *et al.* (1999) first demonstrated that TLR2 is recruited to phagosomes to discriminate pathogens and initiate pro-inflammatory cytokine release (251). Tobian *et al* reported that the 19-kDa lipoprotein of Mtb inhibits phagosome maturation via TLR2 (34). Blander and Medzhitov (2004) further demonstrated that activation signals through TLRs are required for the regulation of phagocytosis (in both the internalisation and phagosome maturation stage) and phagocytosis of bacteria was impaired in the absence of TLR signaling (252).

Avoiding T cell activation by altering antigen processing (34, 36) or inhibiting APC maturation (35) by M.tb components were also reported. The 19-kDa lipoprotein of M.tb inhibited class II MHC (MHC-II) expression and alternate class I MHC (MHC-I) antigen processing via a TLR2-dependent pathway (34). Prolonged exposure (>16 hrs) of human macrophages to LprG, a 24-kDa lipoprotein found in the M.tb cell wall, resulted in marked inhibition of MHC-II Ag processing via TLR-2, though short-term exposure (<6 hrs) to LprG stimulated TLR-2-dependent TNF-α production (36). Mtb-specific IFN-γ producing T cells are essential for T cell-mediated immunity and

IFN-γ is also important for macrophage activation (253). Treating DCs with ManLAM from M.tb was found to cause DC to produce IL-10 instead of IL-12 (41). ManLAM was also found to target DC-SIGN and to down-regulate DC-mediated immune responses by interfering with TLR-mediated signals. Blocking antibodies against DC-SIGN reverse the ManLAM-mediated immunosuppressive effects (35).

Chapter 2. Materials and Methods

1. CLINICAL STUDY

1.1. Patients

Fifteen patients with pulmonary tuberculosis (TB) were recruited at the Royal Free and Middlesex Hospitals, London, UK and underwent lung bronchoalveolar lavage (BAL). Ten out of fifteen patients were also included in the whole blood and cell isolation study (section 1.3 and 1.4). All patients yielded positive cultures from sputum or alveolar lavage fluid, were negative for antibodies to HIV and responded clinically to anti-TB treatment.

1.2. Healthy controls

Controls (n=10) were healthy volunteers matched to the cases for age (within 4 years), gender and ethnicity. They were asymptomatic, had no risk factors for HIV infection (but were not formally tested), and had normal chest x-rays. In order to exclude latent Mtb infection in the controls, T-cell IFN-γ ELISPOT responses to ESAT-6 and CFP-10 peptide pools were determined (T SPOT TB, Oxford Immunotec, England) (254). Six of these volunteers underwent (BAL). Participants with a history of atopy or helminth infection, another inflammatory disorder, pregnancy or on immunosuppressive medication were excluded from the study. Informed consent was obtained from all patients and the Royal Free and UCLH ethical review committees approved the study.

1.3. Processing of whole blood samples

Whole blood (2.5 to 20 ml) was taken before or within the first 2 weeks of anti-TB treatment (baseline) and 2.5 ml was immediately transferred from the patient into PAXgene Blood RNA tubes (PreAnalytix, Qiagen, Cologne, Germany) to fix the mRNA profile (255). The remaining blood was used for further experiments (cell sub-population studies). Samples not immediately fixed in RNA buffer, were transported in a thermos flask at 37°C in accordance with regulations governing the transport of bio hazardous materials. A safety and hazards assessment was conducted far all experiments and the relevant good laboratory practice and category 3 laboratory training courses were undertaken.

1.4. Cell separation to acquire whole blood cell sub-populations

1.4.1. Density centrifugation to obtain mononuclear cells

For the cell separation study, peripheral blood mononuclear cells (PBMCs) were separated from fresh whole blood by density gradient centrifugation. Plasma and PBMCs were separated by gradient centrifugation on Ficoll-Paque. Plasma was stored at -80°C until use. Whole blood was layered (diluted 1:1 with Roswell Park Memorial Institute medium (RPMI)) over Ficoll-Paque Plus (sodium metrizoate/polysaccharide solution; Amersham Biosciences, Buckinghamshire, UK), and centrifuged at 900 *g* for 15 minutes (no brake). The interface PBMC layer was harvested with a Pasteur pipette and transferred into a fresh universal tube. Red cells were lysed by incubation with ammonium chloride solution for 10 minutes on ice (StemCell Technologies, Vancouver, Canada). PBMCs were washed twice in wash medium (serum-free RPMI 1640 medium supplemented with 2mM L-glutamine, 50 U/ml of penicillin, 50 μg/ml of streptomycin) by centrifugation at 400g for 10 minutes at room temperature. The viable

cells were counted by trypan blue exclusion using a Neubauer counting chamber (depth 0.1 mm).

1.4.2. Isolation of cell sub-populations

PBMCs from 10 patients with PTB and their healthy matched controls were used for cell sub-population studies. Enrichment of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ and depletion of T cells was performed using antibody based density centrifugation reagents, added to whole blood, according to the manufacturer's directions (RosetteSep, StemCell Technologies, Vancouver, Canada). The RosetteSep antibody cell separation cocktail consists of monoclonal antibodies to CD surface molecules and a tetrameric glycophorin antibody that agglutinates red blood cells and antibody bound leucocytes. The antibody bound cells are removed by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK). The negative selection T cell cocktail contains glycophorin A, anti-CD16, anti-CD19, anti-CD56 and anti-CD36 antibodies; the CD 4 cocktail in addition contains anti-CD8 antibody; the CD8 cocktail has in addition anti-CD4 antibody; total non T cells are acquired by depleting all $CD3^+$ T cells. Cell viability was checked using trypan blue exclusion.

1.5. Bronchoalveolar lavage

BAL was undertaken under local anaesthetic and midazolam sedation. To obtain BAL fluid 0.9% saline was instilled into a radiologically involved lung segment, using 180 ml saline in 60ml aliquots. In control donors the right middle lobe was lavaged. BAL fluid was transported on ice to a laboratory adjacent to the bronchoscopy suite, filtered through a 100μm Partec filter (CellTrics®, Münster, Germany) and centrifuged at 900 *g*

for 3 minutes. A buffer containing chaotropic salts (RLT buffer, Qiagen) was added to the cell pellet to fix the RNA profile and the sample homogenised to disrupt cell structure (QIA shredder, Qiagen). The supernatant was frozen at -80°C and concentrated ~ 10 fold before analysis (Centriprep YM-3 Centrifugal Filter Unit, Millipore, Watford, England). An aliquot of fresh lavage fluid was used to determine BAL lymphocyte sub-populations by flow cytometry (section 1.6).

1.6. Flow cytometry

Cell sub-population purity and lymphocyte counts in BAL (total $CD3^+$, $CD4^+$ and $CD8^+$) were confirmed by flow cytometry (10^4 gated events). Cells (~ 7.5 x 10^5) were stained with anti CD4-FITC, anti CD8-PE and anti CD3-PercP antibodies (BD TritestTM, BD Biosciences, Oxford, UK) and incubation on ice for 15 minutes. Excess antibody was removed by washing in FACS buffer (1% BSA-0.1% Sodium Azide-PBS) and cells were fixed in 1% formaldehyde. Mean cell purities of the relative whole blood fractions were > 95%, 99%, 93% and 90% for $CD3^+$, $CD3^-$, CD4+ and CD8+ fractions, respectively and were determined by FACS using the Cell Quest 3.3 (FACScan; Becton Dickinson, San Jose, CA) and Ortho Cytoron absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ).

1.7. RNA resources for the clinical study

Samples for the clinical study were processed and kindly given by Dr. Keertan Dheda. The RNA template was qualitatively assessed and quantified using an Agilent Technologies 2100 Bioanalyser (RNA 6000 Nano Labchip® Kit) (section 7.2). 1 ng/reaction of total RNA was reverse transcribed using sensiscript reverse transcriptase (For detail see section 7.3).

2. *In vitro* EXPERIMENTS: THP1

2.1. Cell propagation

The human myelomonocytic cell line THP1 (American Type Culture Collection, Manassas, Va.) was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (Gibco BRL, Paisley, UK) and 10 % fetal bovine serum at 37 °C, 5 % CO_2. The morphology of undifferentiated THP1 cells consists of small clumps with round irregularly sized cells in suspension. For routine laboratory culture, cells were cultured in flasks (Nunc, 15 ml) and propagated twice a week.

2.2. Selection of differentiation agents

Three commonly used differentiation agents, phorbol myristate acetate (PMA), 1,25 (dihydroxy) vitamin D3 and dimethyl sulphoxide (DMSO) were titrated in a pilot study on THP1 cells. Preliminary results suggested that all three agents induced morphological changes to macrophage-like cells with adherence to the bottom of the well after 24 hours in culture. Surface expression of myelomonocytic markers (CD11b and CD14) and cell activation markers (CD80, CD86) was performed on differentiated cells by flow cytometry. Due to limited resources, present study did not include the macrophage marker, CD16. Cells differentiated by the three agents had increased expression of CD11b and CD86, but had no changes in expression of CD80 or CD14. High surface expression of CD86 was found in wells cultured with 1,25 (dihydroxy) vitamin D3. This is consistent with the elevated expression of mRNA encoding TLR2, TLR4 and CD14 that was noted later in THP1 cells differentiated by 1,25 (dihydroxy) vitamin D3 (personal communication with Dr. R. Martinelli). 1,25 (dihydroxy) vitamin

D3 suppresses growth of *M. tuberculosis* in a human macrophage-like cell line (HL60), as first reported by Rook (9, 256, 257), suggesting regulation of gene expression by vitamin D3. DMSO was chosen as differentiation agent for the present study at the concentration of 1.2%.

2.3. Cell differentiation

For the induction of cell differentiation, cells (2x 10^5/ml) were initially propagated in 250 ml flasks (Nunc) for 2 days. The cells were treated with 1.2% of dimethyl sulphoxide (DMSO, Sigma-Aldrich, Dorset) for 24 hours to differentiate them into macrophages (258). DMSO was removed by washing off twice with wash medium (RPMI 1640-Penicillin-Streptomycin) and cells were resuspended to 2 x 10^6/ml in culture medium.

2.4. Monitoring cell growth and viability

Cell growth, viability and contamination were routinely checked by microscopy when passaging cells as well as before harvesting. Cell viability was assessed by trypan blue exclusion based on the assumption that cell membrane integrity represents viable cells.

2.5. Sonicated mycobacterial preparations

Crude whole cell sonicates of *Mycobacterium tuberculosis* H37Rv (ATCC 25618) and *Mycobacterium vaccae* (NCTC 11659) were prepared by the lab manager Mr. Graham McIntyre using a modification of the method of Paul (259). Briefly, *Mtb* H37Rv (ATCC 25618) and the fast growing environmental saprophyte *M. vaccae* (NCTC

11659) were grown in Middlebrook 7H10 agar (Difco) containing 10% v/v OADC supplement (Becton Dickinson, UK) in the category 3 laboratory. Colonies were harvested and centrifuged at 20,000g for 15 min, washed twice with PBS (pH 6.8) and suspended in 50 ml of PBS. The suspensions were sonicated for 15 min in a 100-watt ultrasonic disintegrator with the wave peak distance set at 8-9 μm, then centrifuged at 70,000 g for 30 min to remove cellular debris. The supernatants were then filter-sterilised (0.2 μm-pore size) and the protein concentration quantified by the Warburg and Christian method (260).

2.6. Determination of protein concentration: spectrophotometer

The most common methods for determining the concentration of proteins in solution are the spectrophotometric method: Warburg-Christian method (260) and Bradford method (261). The Warburg-Christian method was used to quantitate the sonicate mycobacterial protein concentration by making a direct absorption measurement of a solution at 260 and 280nm. Proteins exhibit a strong absorption at 280 nm due to the aromatic rings of tyrosine and tryptophan residues. However, nucleic acids absorb strongly at 280 nm too. In order to compensate for contamination with nucleic acids, measurement for nucleic acid concentration is performed at OD260. One can then calculate the ratio E280/E260. Using this ratio and a factor that can be read off in table 2.1., and the dilution factor it is then possible to calculate the protein concntration. The sensitivity of the Warburg-Christian method varies with the tyrosine and tryptophan content of the protein solution, but it is in the range of 1 μg protein/ml to 1 mg protein/ml (http://tecn.rutgers.edu/bio301s/Lab%203-%20protein%20diagram.htm).

The protein concentration is given by:

Protein concentration (mg/ml)

= Extinction at 280nm x Factor (Table 2.2) x dilution factor

Alternatively, a formula can be used for calculation protein concentration:

(1.55 * OD280 - 0.76 * OD260) *dilution factor = protein concentration {mg/ml}

(http://www.protocol-online.org/archive/posts/3685.html)

The sonicates were then aliquoted and stored in -80°C. All the *in vitrol* studies used the same batch of sonicates. The sonicates are denoted sMtb and sMv respectively.

Table 2.1. Factor for the calculation of the protein concentration by Warburg & Christian (1941) (260). Measure the absorbance of a diluted protein solution at both 260nm and 280 nm and calculate the ratio E280/E260. Using this ratio, the proportion of nucleic acid in the protein solution and a factor for the calculation of the protein concentration can be read off from the table. The figures from the table had been calculated from yeast experiment by Warburg & Christian (260).

E280/E260	**Nucleic acid (%)**	**Factor**
1.75	0	1.118
1.60	0.3	1.078
1.50	0.50	1.047
1.40	0.87	1.011
1.30	1.26	0.969

1.25	1.49	0.946
1.20	1.75	0.921
1.15	2.05	0.893
1.10	2.4	0.863
1.05	2.8	0.831
1.00	3.3	0.794
0.96	3.7	0.763
0.92	4.3	0.728
0.90	4.6	0.71
0.88	4.9	0.691
0.86	5.2	0.671
0.84	5.6	0.65
0.82	6.1	0.628
0.80	6.6	0.605
0.78	7.1	0.581
0.76	7.8	0.555
0.74	8.5	0.528
0.72	9.3	0.500
0.70	10.3	0.470
0.68	11.4	0.438
0.66	12.8	0.404
0.64	14.5	0.368
0.62	16.6	0.330
0.60	19.2	0.289

2.7. Cell stimulation

For cell stimulation, 2 x 10^6/ml of differentiated cells was incubated with different concentrations of mycobacterial antigens: 90 μg/ml s*Mtb*, 300 μg/ml s*M. vaccae*, 4.8 μg/ml LPS (Sigma-Aldrich, Dorset, UK) and PBS as negative control and cells were harvested at 6 hrs, 18 hrs, 24 hrs and 48 hrs post-treatment. The concentrations of antigens used in the experiments were optimised in pilot experiments, using IL12p40 cytokine release as the readout.

2.8. Synthetic peptide: Pam3CysK4

In subsequent experiments differentiated THP1 cells were treated with 100 μg/ml Pam3CysK4 (Bachem, Delph Court, UK) or with 90 μg/ml sonicated *Mtb* for various times. The concentrations of antigens used in the experiments were optimised in pilot experiments with titration of Pam3CysK4 at concentrations of 10 μg/ml, 100 μg/ml and 200 μg/ml on THP1 cells.

3. *In vitro* EXPERIMENT: INFECTION WITH LIVING *M. tuberculosis* or *M. vaccae*

3.1. Healthy donors

5 healthy volunteers (mean age 38 years, 4 females, mixed ethnicity- 1 Indian, 1 Korean, 1 Chinese, 1 Philippine, 1 caucasian) participated in the study. Control subjects had previous exposure to *M. tuberculosis* but no latent tuberculosis infection, which was excluded by determining TB antigen-specific (ESAT-6 and CFP-10 peptide pools) IFN-γ ELISPOT responses in PBMCs.

3.2. Suspensions of living mycobacteria

Mtb H37Rv (ATCC 25618), a Mexican clinical isolate (CPA96) and the fast growing environmental saprophyte *M. vaccae* (NCTC 11659) were grown on Middlebrook 7H10 agar (Difco) containing 10% v/v OADC supplement (Becton Dickinson, UK) in the category 3 laboratory. Colonies were transferred into sterile screw top Eppendorf tubes that contained deionised water with 0.1%v/v tyloxapol and four sterile 1.5 to 2 mm glass beads. Mycobacteria were disaggregated by vigorous vortexing with glass beads for 1-2 mins and the tubes were left standing for 5 minutes to allow large aggregates to settle. The supernatant suspension was then removed, centrifuged at 1200 rpm for 2 minutes and diluted 1 in 10 in formaldehyde for counting. 10 μl of the suspensions were added to Improved Neubauer haemocytometers and organisms were allowed to settle for at least 30 mins at room temperature. A direct microscopic count was performed to determine mycobacterial concentration.

3.3. Infection of peripheral blood mononuclear cells

PBMCs from 5 healthy control donors were separated from heparinised blood (50 ml) by Ficoll density gradient centrifugation and cells were reconstituted at $2x10^6$ cells/ml. PBMCs were infected with living mycobacteria at a dose of approximately 1 organism per macrophage (~10% monocytes in PBMC). Cells were cultured in RPMI 1640 supplemented with 5% heat inactivated human AB serum and 1% L-glutamine, without antibiotics at 37°C, 5% CO_2 in a category 3 Lab and harvested at 18 hrs, 24 hrs, 48 hrs and 66 hrs post-treatment. The viability of mycobacteria was assessed by culturing the diluted bacteria on Middlebrook 7H10 agar.

4. RNA HALF LIFE STUDY

4.1. Clinical study

4.1.1. Donors

Eight patients with pulmonary tuberculosis (sputum culture positive, negative for antibodies to HIV, mean age 33 years, 5 males, mixed ethnicity- 4 black Africans, 2 Somalis and 2 Caucasians) and 5 healthy volunteers (mean age 34 years, 3 males, mixed ethnicity- 1 Indian, 1 Korean, 1 Chinese, 2 Caucasians) were recruited at the Royal Free and Middlesex Hospitals, London, UK. Control subjects had previous exposure to *M. tuberculosis* but no latent tuberculosis infection (section 1.2)

4.1.2. Inhibition of RNA transcription

All blood samples were treated with 5 μg/ml Actinomycin D (ActD, Sigma-Aldrich, Dorset) and samples harvested at 0, 60, 120 and 240 minutes. The optimum ActD

concentration was titrated (5, 10 and 20 μg/ml) in pilot experiments using cells from control donors and the results suggested that 5 or 10 μg/ml gave similar effects on rates of RNA degradation. Hence, 5 μg/ml of ActD was chosen for the clinical study. To verify that ActD effectively blocks transcription activity, Myc-proto-oncogene protein (*c-myc*) transcription factor p64 was selected as positive control (262).

4.2. *In vitro* THP1 study

4.2.1. Inhibition of RNA transcription

To measure mRNA half-life in THP1 cells, differentiated cells (2 x 10^6/ml) were incubated with or without 90 μg/ml sonicated Mtb for 6 hours (details can be seen in section 2). Six hours incubation with sMtb increased expression of the gene encoding TLR2 as well as release IL-12p40 measured by ELISA. Both stimulated and unstimulated cells were treated with or without 10 μg/ml ActD and cells were harvested at the indicated times for assay of the genes of interest by RT-PCR. Optimal concentrations of ActD for use in THP1 cell cultures had been titrated in pilot experiments. The effect of ActD on blocking transcription activity was assessed using Myc-proto-oncogene protein (*c-myc*) transcription factor p64 as positive control (262).

4.2.2. p38 MAPK pathway: p38 antagonist preparations

To check whether changes in mRNA half-life were p38-MAPK-dependent, differentiated THP1 cells were treated with 90 μg/ml sonicated Mtb for 6 hrs. Cells were then treated with 10 μg/ml ActD with or without 5 or 10 μM of p38 antagonist (SB202190) (Calbiochem, Nottingham, UK). Osteogenic differentiation factor (SOX 9) gene expression was used as a positive control for the effects of SB202190 as the half-life of its mRNA is known to be regulated by p38 in THP1 cells (263).

5. MOLECULAR BIOLOGY

5.1. Characterisation of novel splice variant of TLR1

5.1.1. Primer design

During the initial screening, a splice variant of TLR1, designated hsTLR1, was discovered in THP1 cells using the TLR1-1-3 primers (Table 2.2). The original TLR1-1-3 primers targeted exons 1 and 3 of the TLR1 gene. This gave rise two PCR products, the designed amplicon of 142 bp and a smaller amplicon of 65 bp which omitted exon 2 (Figure 2.1. A.1). New primers were designed to distinguish the novel splice variant of TLR1. The 5' primer for the TLR1 splice variant spanned the exon 1-exon 3 junction which would only anneal with cDNA lacking exon 2 (Figure 2.1. B). To specify the original TLR1 cDNA the sense primer targeted exon 1 while the antisense was located on exon 2, generating a 115 bp amplicon only from the exon 2-containing TLR1 (Figure 2.1. A.1). Primer sequences can be seen in Table 2.2.

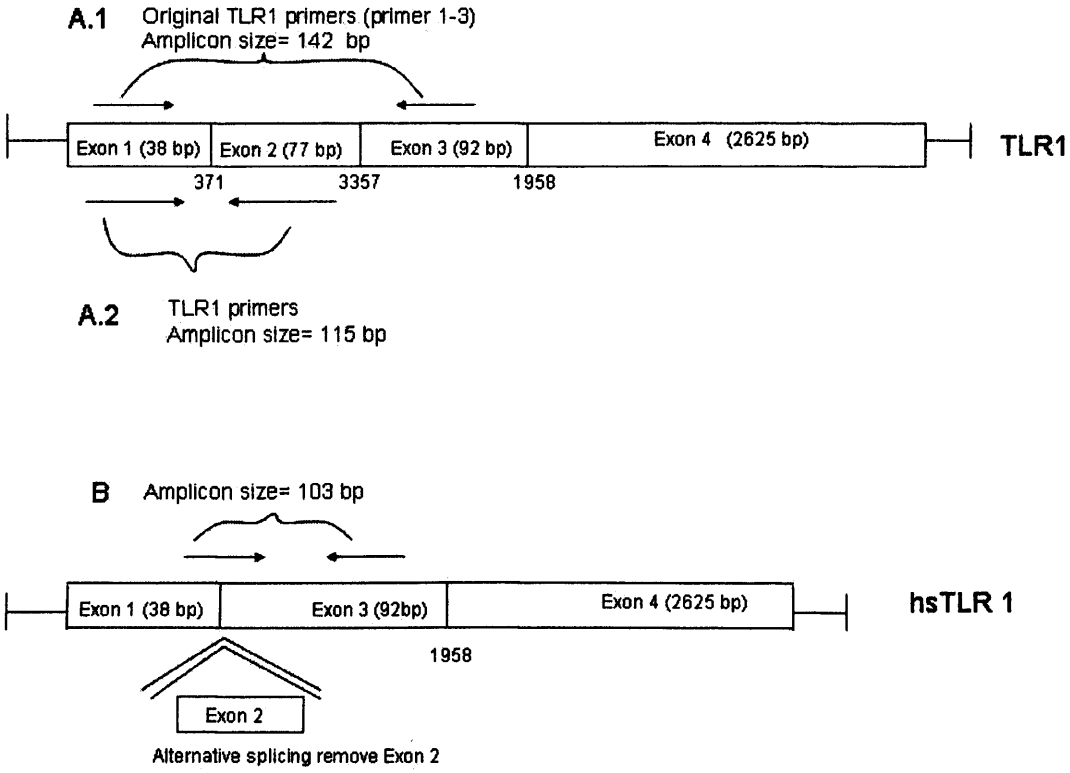


Figure 2.1. Specificity of primers for TLR1 and hsTLR1.

The 5' primer for the hsTLR1 specific PCR spanned the exon 1-exon 3 junction which would only anneal with cDNA lacking exon 2 (Figure 2.1. B). The sense primer for TLR1 was sited on exon 1 whereas the antisense was located on exon 2, generating a 115 bp amplicon that contains exon 2 transcript (Figure 2.1. A.1)

5.1.2. Agarose gel electrophoresis

2 µl of PCR products were run on 1.8% agarose gel (1x SYBR Green I dye) with 1 µl of DNA Mass Ladder (Invitrogen, Paieley) by electrophoresis in TAE buffer at 88 V for 1 hour and visualised UV transilumination (Molecular probes, Invitrogen, Paisley, UK). Amplicon size and purity were confirmed by low DNA Mass Ladder, of which each band contains a known weight of DNA in ng as well as a known size in base pairs.

5.1.3. PCR product purification: QIAquick Gel extraction

To separate the two TLR1 amplicons the respective DNA fragments were excised from the agarose gel with a clean, sharp scalpel and purified according to the manufacturer's instruction (QIAquick Gel extraction, QIAGEN, West Sussex, UK).

5.1.4. Estimation of amplicon concentration

The concentrations of purified hsTLR1 and TLR1 PCR products were estimated using the low DNA mass ladder and 1.8% Agarose gel electrophoresis (Invitrogen). Amplicon size and purity were confirmed using the low DNA Mass Ladder.

5.1.5. Ligation: pGEMT TA cloning

Purified PCR products were cloned into the pGEM®-T vector following the manufacturer's instructions (Promega). Briefly, purified PCR products were ligated in 10 µl of 2x rapid ligation buffer reaction (2.5 µl ligation buffer, 0.5 µl pGEM®-T vector, 0.5 µl PCR product, 0.5 µl T4 DNA Ligase and deionised water) at 4 °C overnight.

5.1.6. Transformation

Ligated plasmids were transformed into JM109 competent cells (Promega) by heat-shocking the cells at 42 °C in a water bath for 50 seconds. The cells were then mixed with 500 µl SOC medium (Sigma) and incubated for 1 hour at 37 °C at 150 rpm. 100 µl of SOC culture was spread onto an LB agar plate (100 µg/ml Ampicillin, 0.5 mM IPTG, 80 µg/ml X-gal) and incubated at 37 °C overnight. Transformed cells were selected using the ampicillin resistance conferred by the vector. Successful insertion of amplicon was identified by colour screening. Insertion of the amplicon interrupts the coding sequence of β-galactosidase that would normally result in a blue colour. White transformed colonies were picked and inoculated in 1-10 ml of LB broth (100 µg/ml Ampicillin) and grown overnight at 37°C in a rotary shaker (225 rpm).

5.1.7. Plasmids extraction: Wizard Plus Minipreps

Plasmids from bacterial cultures were extracted using the Wizard Miniprep kit (Promega) following the manufacturer's instructions. 10 µl of plasmid DNA were digested with restriction enzyme (2 µl RE 10x buffer, 0.2 µl Acetylated BSA, 0.5 µl *Eco* RI enzyme) (Promega) at 37 °C for 2 hours. The product sizes of plasmid DNA were confirmed by electrophoresis in 1.8% TAE Agarose gel (Sybr Green I) and 1Kb ladder (Promega). Frozen stocks of correctly inserted bacterial cultures were taken by adding 20% glycerol and storing at -80 °C.

5.1.8. Sequencing of inserts

Sequences of inserted amplicons were obtained using the ABI PRISM® 3100 Genetic Analyzer.

5.2. Construction of standard curves

Construction of plasmid DNA for standard curves followed the methods described in section 5.1. Primers used for amplified PCR products were listed in Table 2.2 (section 7.4). There are a number of ways to estimate the concentrations of plasmid DNA (or PCR products). Firstly, concentrations of purified plasmid DNA (or PCR products) were estimated by comparison with the low DNA mass ladder after 1.8% Agarose gel electrophoresis (Invitrogen). Each band contains a known weight of DNA in ng, as well as a known size in base pairs. The fluorescent units for each band were plotted against its weight to create standard curve. This standard curve was used to extrapolate the number of amplicon copies in the plasmid DNA (or PCR samples). Once the copy number of each plasmid DNA (PCR products) was determined, a standard curve could be diluted down for quantification purposes.

5.3. Characterisation coding sequences: cDNA cloning

5.3.1. Primer design

Specific primers were designed to distinguish coding sequences (CDS) of hsTLR1 (Figure 5.2.). The 5' primer for the TLR1 splice variant spanned the exon 1-exon 3 junction which would only anneal with cDNA lacking exon 2 (Figure 5.2. B) whereas the 5' primer for TLR1 was sited on exon 1 (Figure 5.2. A). The 3' primer for TLR1 and hsTLR1 was located at same region of 3'-UTR, generating amplicons of 2943 bp and 2848 bp, respectively. Primer sequences are shown in table 1 (section 7.5).

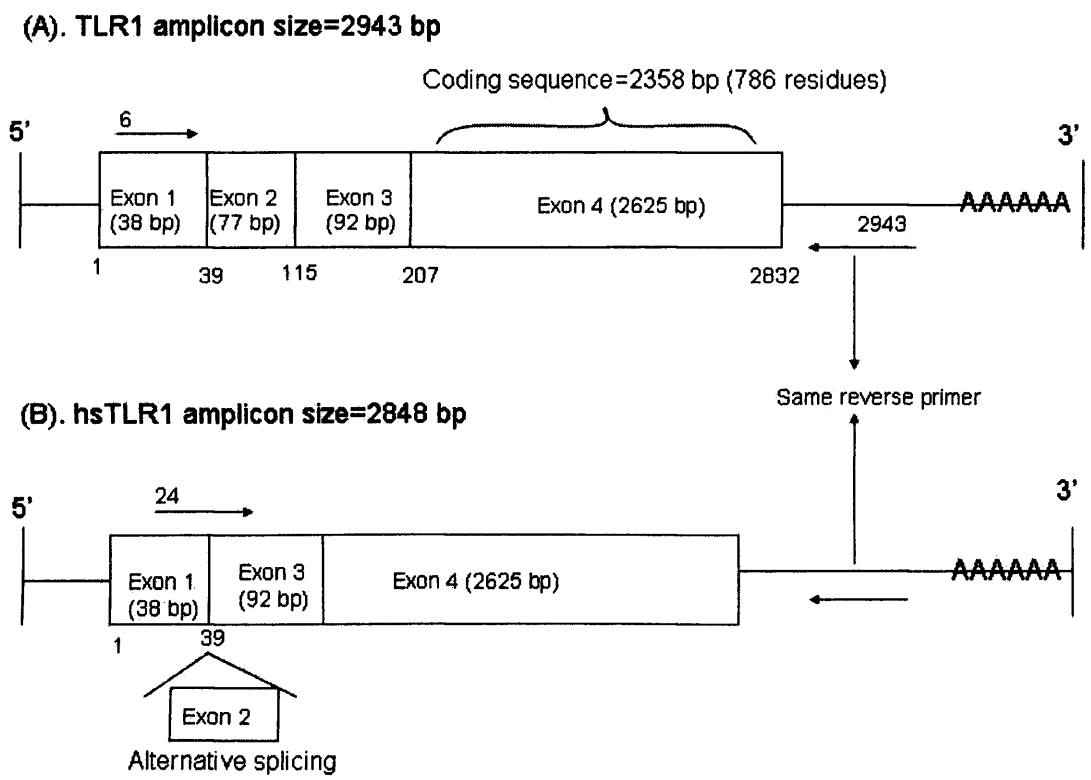


Figure 2.2. Diagram of the primer positions and respective sizes of amplicons of TLR1 (A) and hsTLR1 (B). The 5' primer for the TLR1 splice variant spanned the exon 1-exon 3 junction which would only anneal with cDNA lacking exon 2 (Figure 2.2. B) whereas the 5' primer for TLR1 was sited on exon 1 (Figure 5.2. A). The 3' primer for TLR1 and hsTLR1 was located at same region of 3'-UTR, generating amplicons of 2943 bp and 2848 bp, respectively. Primer sequences are shown in table 1 (section 7.5).

5.3.2. Touchdown PCR

TLR1 and hsTLR1 were amplified using Advantage-HF 2 polymerase (Advantage™ HF 2 PCR Kit, BD Bioscience, Oxford). 25 µl PCR reactions were performed using 5 nM TLR1 or hsTLR1 specific forward primers; a common reverse primer was used for each reaction (Figure 5.2.). The optimum ratios of the two buffers differed for each primer set. hsTLR1 (0.83 µl 10x Advantage 2 PCR buffer, 1.67 µl 10x HF 2 PCR buffer) and TLR1 (2.5 µl 10x Advantage 2 PCR buffer, 2.5 µl 10x HF 2 PCR buffer). PCR reactions were performed using touchdown PCR: 94°C, 1 minute; 31 cycles of 94°C, 30 sec & 62°C to 55°C for 1 minute; 72°C for 3 minute.

5.3.3. PCR product purification: Wizard SV Gel clean-up

Fresh PCR products were purified using Wizard® SV Gel and PCR Clean-Up kit according to the manufacturer's instructions (Promega). The remaining salt content in the purified PCR product had a great effect on the ligation of hsTLR1. Successful ligation only occurred when excess salt or quanidine isothiocyanate (membrane binding solution) were removed using Wizard® SV Gel and PCR Clean-Up kit.

5.3.4. Estimation of PCR product size

Purification of the correct PCR products was confirmed by electrophoresis in 0.7% TAE Agarose gel (SYBR Green I). The concentrations of hsTLR1 and TLR1 PCR products were estimated by low DNA mass ladder (Invitrogen, Cat. No.10068-013).

5.3.5. Ligation

Purified PCR products were TA cloned into pCR®4-TOPO vector following the manufacturer's instructions (Invitrogen). The ligation molar ratio was calculated and molar ratios of 3:1, 1:1 and 1:3 were chosen for the ligation (http://www.promega.com/biomath/default.htm).

5.3.6. Transformation

Ligated plasmids were transformed into "One Shot" chemically competent cells following the manufacturer's instructions (Invitrogen). 2 µl of ligation reaction was transformed into competent cells by heat-shocking the cells at 42 °C in a water bath for 30 seconds. The cells were then mixed with 250 µl SOC medium and incubated for 1 hour at 37 °C at 150 rpm. 50 µl of SOC culture was spread onto an LB agar plate (50 µg/ml Ampicillin) incubated at 37 °C overnight. The pCR®4-TOPO vector allows direct selection of recombinants via disruption of the lethal E.coli gene, *ccdB*. Ligation of a PCR product disrupts expression of the *lacZα-ccdB* gene, allowing growth of transformed competent cells whereas cells that contain non-recombinant vector are killed by expression of the lethal gene. Transformed colonies were picked and inoculated in 1-10 ml of LB broth (50 µg/ml Ampicillin) and grown overnight at 37°C, in a rotary shaker (225 rpm).

5.3.7. Restriction digestion

10 µl of plasmid DNA were digested in restriction enzyme buffer (2 µl RE 10x buffer, 0.2 µl Acetylated BSA, 0.5 µl *Eco* RI enzyme) (Promega) at 37 °C for 2 hour. The product sizes of plasmid DNA were confirmed by electrophoresis in 0.7% TAE Agarose gel (SYBR Green I) and 1Kb plus ladder (Invitrogen). Correct recombinant bacterial cultures were frozen by adding 20% glycerol and storing at -80 °C.

5.3.8. Plasmid extraction: FastPlasmid Mini

Protein and genomic DNA of plasmids from bacterial cultures were extracted using FastPlasmid Mini kit (eppendorf) following manufacturer's instructions.

5.3.9. Sequencing of inserts

200 μg of plasmid DNA in 7 μl were amplified by M13 forward and reverse primers for sequencing using the ABI PRISM® 3100 Genetic Analyzer.

5.4. RNA secondary structure

RNA secondary structure was predicted by using the Mfold web server (http://www.bioinfo.rpi.edu/~zukerm/rna/).

6. IMMUNOASSAY: ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Sandwich ELISAs kits for IL-12p40 (DuoSet ELISA Development, R&D), IL-4 and IFN-γ (both from Pelikine CompactTM, Sanquin reagents, Amsterdam, The Netherlands) were performed according to the respective manufacturers' instructions. The concentrations of antigens (sM.tb, sM.*vaccae* and LPS) used in the experiments were optimised in pilot experiments, using IL-12p40 cytokine release as the readout. IL-4 and IFN-γ serum levels were measured in peripheral blood mononuclear cells cultured with living organisms (chapter 7). The sensitivity of the assays was 16.625 pg /ml (IL-12p40), 0.5 pg/ml (IL-4) and 5 pg/ml (IFN-γ). Plates were read using an ELISA-plate reader (MR5000, Dynatech, Guernsey). Standard curves were plotted and sample protein concentrations were calculated using Graphpad Prism.

7. REAL-TIME PCR

7.1. RNA extraction

Total RNA was isolated from whole blood using the Paxgene Blood RNA Kit (PreAnalytix, Qiagen) and from purified or cultured cells using the RNeasy® Mini Kit (Qiagen, West Sussex, UK). Cells were harvested at each time point; after centrifugation, pellets were lysed immediately in RLT buffer and supernatants were kept in a –20 °C freezer. This immediate lysis prevents unwanted changes in RNA concentration that may occur if there is a delay. After disruption of cell walls and plasma membranes by the lysis buffer, a QIAshredder spin column was used to homogenise the sample by centrifugation for 2 minute, which can reduce the viscosity of the cell lysates. Both the disruption and the homogenisation stage are vital for the optimal yield of total RNA. Extracted RNA was treated with DNase (Qiagen).

7.2. RNA quality assessment

The RNA template was qualitatively assessed and quantified using an Agilent Technologies 2100 Bioanalyser (RNA 6000 Nano Labchip® Kit). 1 µl total RNA was pippetted into an RNA 6000 nano chip or RNA 6000 pico chip (Agilent Technologies). Also included was a lower marker. This allows for sample alignment, size estimation and distinguishes different types of RNA based on the electrophoretic traces. The contamination of genomic DNA, or degraded RNA also showed on the electrophoretic traces or gel bands. It also simultaneously assesses RNA concentration. The Total RNA was aliquoted and stored at –80 °C in a freezer.

7.3. Reverse transcription

A fixed amount of total RNA (1 μg /reaction for culture experiments and 25 or 50 ng/reaction for whole blood and cell subpopulation studies) was incubated with 1.5 μM oligo (dT) primer (Promega, Southampton, UK) at 75 °C for 5 minutes to denature RNA. The reverse transcription master mix for the culture experiments was (10x buffer RT, 0.5mM dNTP, 10 Units RNase inhibitor, 4 Units Omniscript reverse transcriptase and RNase-free water) (Qiagen, West Sussex, UK). Sensiscript reverse transcriptase was used for the whole blood and cell subpopulation study because of limited template availability (10x buffer RT, 0.5 mM dNTP, 10 Units RNase inhibitor, 10 Units Sensiscript reverse transcriptase and RNase-free water) (Qiagen, West Sussex, UK). Denatured RNA was added to 20μl RT reaction mix and incubated at 37 °C for 1 hour, followed by 93°C for 5 minutes to denature the enzyme. The cDNA was aliquoted and used for quantitative PCR.

7.4. Primer design and primer sequences

Primers and probes for each gene studied were designed with the assistance of primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the details of primer and probe sequences and working concentrations are shown in table 2.2. Primers were synthesised by MWG (MWG-BIOTECH, Germany) and hydrolysis (Taqman) probes were synthesised by Sigma-Genosys. Primer and probe concentration and annealing temperatures were optimised for optimal efficiency (>95 % assessed using clone amplicon dilution series (Chapter 3, section 2.3 & 3.1) (264).

Table 2.2. Primer and probe sequences used to quantify gene expression by real-time PCR.

Genes	Ensembl transcript ID	Primer/probe sequence	Amplicon (base pairs)	Tm	Concentration of primer/probe
HuPO	ENSG00000089157	Probe: TGCCAGTGTCTGTCTGCAGATTGG Primer: sense-5'-GCTTCCTGGAGGGTGTCC-3' Antisense-5'-GGACTCGTTTGTACCCGTTG-3'	105	55	Primer: 0.5μM Probe: 0.3 μM
GAPDH	ENSG00000111640	Probe: ATTGGGCGCCTGGTCACCAG Primer: sense-5'- GAGTCAACGGATTTGGTCGT Antisense-5'-AATGAAGGGGTCATTGATGG-3'	95	55	Primer:0.4 uM Probe:0.32 uM
β-Actin	ENSG00000075624	Primer: sense-5'-TCAAGATCATTGCTCCTCCTGAG Antisense-5'-CTCCTGCTTGCTGATCCACA	104	59	0.4 uM
TLR1-1-3	ENS00000308979	Primer: sense-5'-CCAAATGGAACAGACAAGCA-3' Antisense-3'-AGGGCCTGGTACCCTATTA-3'	142	55	0.5 μM
TLR1	ENS00000308979	Primer: sense-5'-CTGCCAAATGGAACAGACAA-3' Antisense-5'-GCCACAAAAACAGAAGAGCTG-3'	115	52	0.5 μM
hsTLR1	ENS00000308979	Primer: sense-5'-AAGCAGGTTGTCTTGGGTCTT-3' Antisense-5'-TTGTAGGGGTGCCCAATATG-3'	103	55	0.4 μM
TLR1-cDNA	ENS00000308979	Primer: sense-5'-CTGCCAAATGGAACAGACAA Antisense-5'-GTGAACTGCGACCCGAAG	2943	62-55	0.5 μM

hsTLR1 -cDNA	ENS00000308979	Primer: sense-5'-AAGCAGGTTGTCTTGGGTCTT Antisense-5'- GTGAACTGCGACCCGAAG	2848	62-55	0.5 μM
TLR2	ENS00000260010	Primer: sense-5'-GGAGTTCTCCCAGTGTTTGG-3' Antisense-5'-GCATTGTCCAGTGCTTCAAC-3'	105	56	0.5 μM
TLR4	ENS00000307072	Primer: sense-5'- AGTGAGGATGATGCCAGGAT-3' Antisense-5'-TTCATGCCAGCTCTTCTGTG-3'	147	58	0.4 μM
TLR6	ENS00000308993	Primer: sense-5'- TATCTCAGGATGGTGTGCCA-3' Antisense-5'-CTTTTCACCCAGGCAGAATC-3'	134	57	0.5 μM
TLR7	ENS00000200806	Primer: sense-5'-GGAAATTGCCCTCGTTGTTA-3' Antisense-5'-CTTTTCACCCAGGCAGAATC-3'	102	57	0.5 μM
TLR9	ENS00000310209	Primer: sense-5'-GAAGGGACCTCGAGTGTGAA-3' Antisense-5'-5'-CTGCACCAGGAGAGACAGC-3'	136	56	0.5μM
c-Myc	ENS00000259523	Primer:sense-5'-TTCGGGTAGTGGAAAACCAG-3' Antisense-5'-CAGCAGCTCGAATTTCTTCC-3'	203	56	0. 5uM
SOX 9	ENSG00000125398	Probe: CTCCTCCACGAAGGGCCGCT Primer: sense-5'-AAGCTCTGGAGACTTCTGAACG Antisense-5'- GTAATCCGGGTGGTCCTTCT	105	56	Primer: 0.5μM Probe: 0.3 μM
IL-4	ENSG00000113520	Probe: CTGTAGAACTGCCGGAGCAC Primer: sense-5'-AAACCTTCTGCAGGGCTGCGAC	71	56	Primer: 0.5μM Probe:0.25 μM

		Antisense-5'-GCTGCCTCCAAGAACACAAC			
IL-4δ2	ENSG00000113520	Probe: CTGTAGAACTGCCGGAGCAC Primer: sense-5'- AAACCTTCTGCAGGGCTGCGAC Antisense-5'-GCCTCACAGAGCAGAAGAACAC	76	56	Primer:0.5 μM Probe:0.25 μM
IFN-γ	ENSG00000111537	Probe: TGGCTGTTACTGCCAGGACCCA Primer: sense-5'-TTCAGCTCTGCATCGTTTTG Antisense-5'-TCCGCTACATCTGAATGACCT	112	55	Primer: 0.5μM Probe:0.25 μM

7.5. Amplification profile of Real-time PCR

Human acidic ribosomal protein (HuPO, also known as 60S acidic ribosomal protein P0 (RPLP0)) was selected as reference gene in this study due to its stability in response to Mtb treatment (Details are discussed in chapter 3, section 3.4.3) (6, 45). RNA expression profiles of both target and reference genes were performed using the RotorGene (Corbett research, Sydney Australia) as 12.5 μl / PCR reaction (optimised primer/probe concentrations, set amount of RNA converted cDNA, QuantiTect RT-PCR Master Mix buffer). Reporters where either the non-specific DNA binding dye or a specific hydrolysis probe using QuantiTect SYBR Green or QuantiTect probe Master mix respectively (both from Qiagen). Amplification of the correct product size was confirmed by size using agarose gel electrophoresis (1.8% TAE Agarose gel and 1x SYBR Green I dye) (Molecular probes, Invitrogen, Paisley, UK).

In summary

- All reactions were run in duplicate (12.5µl volume) with 8 serial dilutions of plasmid DNA (standard curve, R^2=0.99 & Efficiency >90%), non-template controls, and containing 1 ng or 1 µg of reverse transcribed RNA for cell subsets/ whole blood samples and for cultured cells, respectively.

- Reporters where either the non-specific DNA binding dye Sybr green or a specific hydrolysis probe using QuantiTect SYBR Green or QuantiTect probe Master mix respectively (both from Qiagen).

- Each run comprised an initial step of 95°C for 15 minutes followed by 40 cycles at 95°C for 5s, optimised primer annealing temperature (see table 2.1) for 10 seconds and 72°C for 20 seconds.

- Primers were intron spanning to avoid amplification of genomic DNA and designed according to recommended guidelines ref.

- Primer and probe concentrations were optimised to achieve efficiencies > 95% for all amplicons. All amplicons were run on an agarose gel (appendix 4) to exclude primer dimers.

- The minimum reproducible detectable threshold was ~10 copies for probe-based target gene amplification (IL-4, IL-4δ2 and IFN-γ) and ~100 copies for SYBER GREEN I based target gene amplification (TLR1, hsTLR1, 2,4,6,7,9). Copy numbers were regarded as valid if replicates were within 30% of each other.

8. FLOW CYTOMETRY

Antibodies directly conjugated to fluorochromes were used to label surface antigens expressed on the THP1 cells. The fluorochromes used were fluorescein-isothiocyanate (FITC) and phycoerythrin (PE), which emitted at standard wavelength 488 nm lasers. The following conjugated antibodies against surface antigens were used: CD14-FITC, CD11b-PE, CD80-PE, CD86-PE (all from BD); TLR1-PE and TLR2-FITC (both from eBioscience). Isotype-matched antibodies were used as controls for non-specific antibody binding and were purchased from the same companies.

Antibody titration was performed prior to the test. 100,000 THP1 cells were washed once with FACs buffer (1% BSA-0.1% Sodium Azide-PBS) by centrifugation at 400g for 10 minutes in a cold room. Supernatant was discarded and cells were stained with specific Antibody or isotype control for 30 minutes on ice in the dark. Unbound antibody was washed off twice by FACs buffer and cells were fixed in 2% paraformaldehyde solution. The cells were analysed immediately or within 24 hours. One colour or two colour staining was performed and analysed using a Becton Dickinson FACSCalibur using CellQuest software, version 3.0.1.

9. DATA ANALYSIS

The Mann-Whitney U test was used for the analysis of differences in the clinical study. Time-course experiments were analysed by 2-way ANOVA with Bonferroni's correction. One phase exponential decay was employed to analyse RNA degradation rates. Analysis was conducted using GraphPad Prism 4 and Excel. RNA secondary structure was predicted by using the Mfold web server (http://www.bioinfo.rpi.edu/~zukerm/rna/).

Chapter 3: Validation of Real time RT-PCR for gene quantification

1. INTRODUCTION

In comparison to other members of the pattern recognition receptor (PRR) family, TLRs are biologically active at low levels and have a high affinity for ligand binding (265). This is reflected in low mRNA copy numbers in human cells (203). TLR have characteristic of tissue distributions, ligand-specificity and control of gene-induction (Chapter 1, section 3.3), and regulation of their genes is very complex. Indeed, recent evidence suggests that TLR expression is controlled at multiple stages, which also differ between species and cell types (247, 266). Surface distributed TLR (e.g. TLR2, TLR4 and TLR2 co-receptors) are expressed at high levels in myeloid lineage cells whilst intracellular receptors (e.g. TLR9) are more likely to be restricted to specific cell types (e.g. pDC and B cells) (203). It was uncertain whether TLRs were expressed in T cells but recent views have been that TLRs are expressed in lymphocytes with low mRNA copy numbers (201, 202, 227, 267). Surface TLR protein can be rapidly down-regulated by distinct mechanisms triggered by the appropriate ligand (268, 269). Inter-donor variation is also a common feature of TLR which may be due to polymorphisms or alternative spicing (27, 28, 31, 250).

Based on these observations, the present study focused on measuring the mRNA level of TLR instead of protein as the first step towards investigation. There are several commonly used methods for quantification of mRNA and each method has its own strengths and weaknesses (Table 3.1). The most desirable method would depend mainly on the aims of the study itself. The aim of this project was to characterise gene

expression profiles of TLR in different cell types in patients with active pulmonary disease. For this purpose real time RT-PCR was chosen as it allows direct comparison of gene expression patterns in different sample populations.

Table 3.1. Comparison of methods of quantification of transcription.

Method	Advantages	Disadvantages
Northern Blotting	- information of mRNA size, alternative splicing and the integrity of RNA samples	- less sensitive - unable to discriminate between related mRNA of similar size
RNAse protection	- mapping transcript initiation, termination sites and intron/exon boundaries - able to discriminate between related mRNA of similar size	- less sensitive
in situ hybridisation	- the only one method that allows localization of transcripts to specific cells within tissue	- low sensitivity
RT-PCR	- most sensitive - to characterize patterns of mRNA expression - to compare the levels of mRNAs in different samples - to discriminate between closely related mRNAs - to analyse RNA structure	- false result by contamination - relatively expensive (e.g. reagent and instrument) - labour intensive (e.g. cloning, optimization of assay)

	- Large dynamic range	
cDNA array	- allows simultaneously studying a group of genes at the same time	- expensive - Less sensitive - Lower dynamic range

1.1. RT-PCR: Overview of PCR amplification

The reverse transcription-Polymerase Chain Reaction (RT-PCR) was first reported by K. Mullis and colleagues in 1985 (270) and this assay relies on the successful application of two separate enzymatic activities. The first activity results in the generation of a DNA molecule that is complementary to the target RNA, which is termed complementary DNA (cDNA). The RNA-dependent DNA-polymerase generates the cDNA and a hybrid-dependent exoribonuclease (RNase H) specifically degrades the RNA in RNA:DNA hybrids. The second enzymatic activity is a heat stable DNA-polymerase that is used to amplify the target gene by primer-specific exponential amplification of cDNA using a thermal cycler.

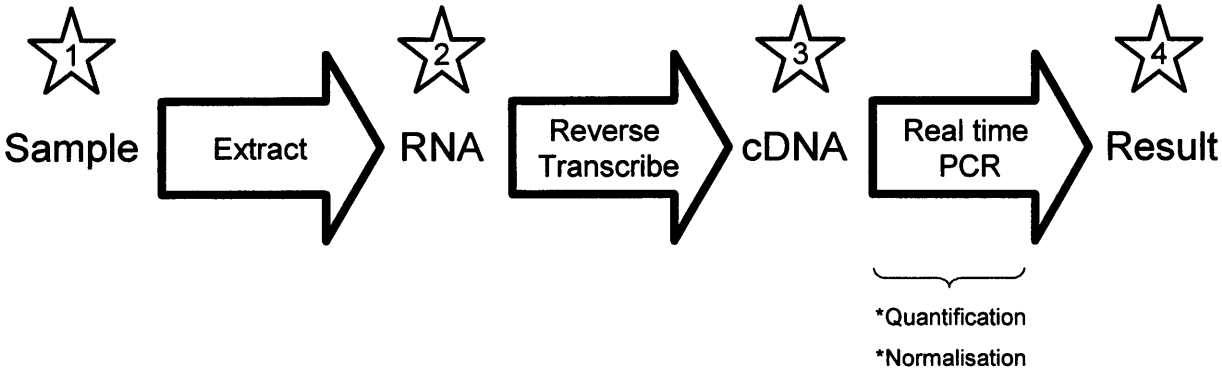


Figure 3.1. Flow chart describing the steps involved in RT-PCR. Ensuring RNA quality prior to RT is the key factor for a successful PCR operation. Step 1 and 2: Cells are harvested and RNA is extracted. Integrity and concentration of total RNA are assessed by Bioanalyzer. Step 3: RNA is reverse transcribed to cDNA by RNA-dependent DNA polymerase (1st enzymatic activity). Step 4: Prior to the final quantification of the gene of interest, the assay must be optimized for optimum amplification efficiency and normalized to control the errors during steps 1 to 4 (2nd enzymatic activity).

There are four stages during a normal PCR amplification (illustrated in Figure 3.2) which are dependent on the amount of template and rate of increase of that product with each cycle. The initial stage is a logarithmic increase of target template that cannot be measured because DNA concentration is too low for detection, which is termed the linear ground phase. The second stage is also during the logarithmic amplification of the template but the DNA concentration is high enough to be measured. Of note, the PCR cycle at which the linear ground phase enters the exponential phase depends on the starting template concentration and the methods used for detecting the product (e.g. hydrolysis probe v.s non-specific DNA-binding dye such as SYBR Green). During the 3rd phase the amplification efficiency is log-linear.

The final plateau phase is characterised by a sharp drop in amplification due to the dynamics of competition between the primers and the templates. There is little relationship between DNA input and amplified target at this stage. Recording the fluorescent emission signal during the exponential amplification stage improves PCR from the conventional qualitative to a quantitative assay.

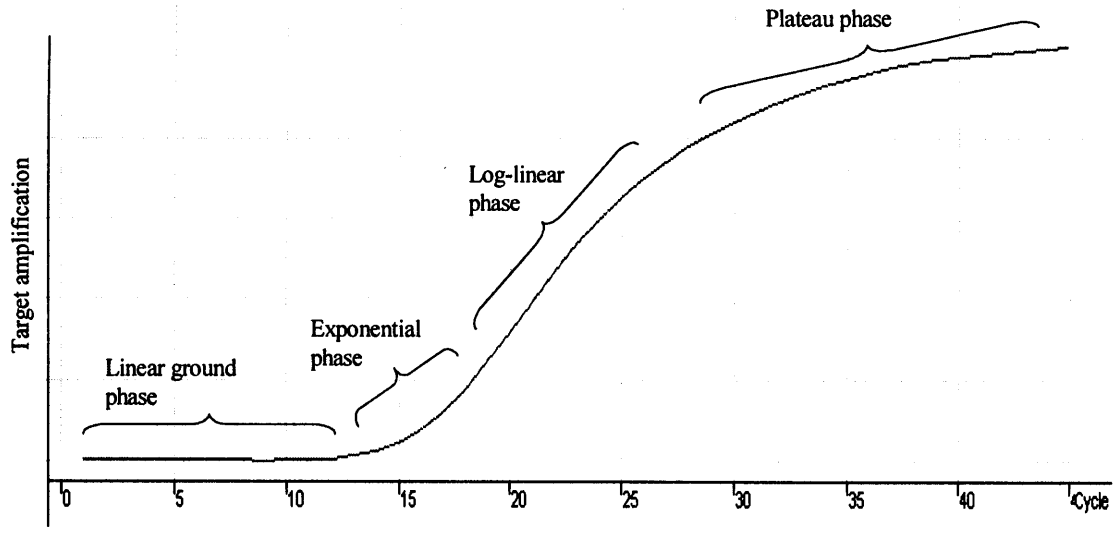


Figure 3.2. Kinetics of PCR amplification. During the linear ground phase, there is relatively little increase in absolute amount of template. In the 2nd phase there is exponential amplification; only here is the amount of amplified target directly proportional to the input amount of target RNA. During the 3rd phase the amplification efficiency is log-linear. The final plateau phase is characterised by a sharp drop in amplification due to reagent and template limitation; there is little relationship between RNA input and amplified target.

1.2. Quantitative PCR

Quantitative that PCR detects amplicon in the exponential phase was developed to facilitate the study of specific nucleic acid. This resulted in the possibility of directly comparing results between experiments and improving reliability and reproducibility. Real time RT-PCR can exploit better chemistries compared to conventional PCR (double stranded DNA-bound SYBR Green fluorescent dye v.s. amplicon specific hydrolysis probe) and better quantification by improving amplification efficiency. The principle of quantitative PCR is to measure target gene expression relative to an internal control with either known or unknown concentration. The internal control normally refers to a house keeping gene (reference gene).

1.2.1. Quantification method: absolute vs relative quantification

There are two ways to quantify mRNA transcripts: absolute and relative quantification. Absolute quantification allows the determination of target copy number per cell or per million copies of reference gene or relative to total RNA concentration by calibration to a standard curve generated in the same run. By contrast, relative quantification measures the relative change in mRNA expression levels as an arbitrary unit. Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of a housekeeping gene. The main problem of relative quantification is that it cannot determine PCR efficiency. This creates a problem in inter-assay comparison of the results between different laboratories.

1.2.2. Absolute quantification: standard curve

The calibration curves used in absolute quantification are normally based on known concentrations of molecules, e.g. recombinant plasmid DNA, genomic DNA, RT-PCR product and recombinant RNA. Stability and reproducibility in kinetic RT-PCR depends on the type of standard used as well as on careful assay optimisation. PCR products are commonly used because they are easily synthesised. However PCR product is difficult to dilute accurately at low concentration because of contamination by primer artefacts. This problem can be avoided by the use of cloned plasmid DNA. Cloned DNA and genomic DNA are very stable and generate highly reproducible standard curves even after a long storage time. A second advantage of cloned DNA is an accurate knowledge of its concentration and length. A problem with DNA-based calibration curves is that they only measure the PCR step, unlike the mRNA of interest that must first be reverse transcribed. Therefore, quantification with external standards requires careful optimization of its precision (replicates in the same kinetic PCR run – intra-assay variation) and reproducibility (replicates in separate kinetic PCR runs – inter-assay variation) in order to understand the limitations of the assay.

1.3. Normalisation of RT-PCR

After accurate quantification, normalisation of samples is also required to see the change of specific gene expression between samples. Misinterpretation of data may occur when comparing different individual samples, mainly due to the variation in the amount of starting material between samples. Samples can be normalized with cell number, total RNA or house keeping genes (reference genes). Normalisation with cell number and total RNA acts as an external control but fails to provide internal controls for Reverse Transcription or PCR efficiency and cells may proliferate during the

experiment (271). House-keeping genes by definition are genes essential for cell functions and cell survival. In theory they should be expressed at constant levels regardless of experimental conditions. Based on this assumption, they were selected as endogenous controls for real time RT-PCR but their stability under experimental conditions has rarely been checked. Although house-keeping gene normalisation has been the favoured method for some time, there is an increasing concern in the literature with regard to the use of unvalidated house keeping genes, in particular β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (272-274). The term 'house-keeping gene' was therefore regarded as inappropriate and 'reference gene' was proposed instead.

The aims of this chapter are to optimise factors that have an effect on the reproducibility of quantitative PCR assay:

(1) RNA quality

(2) Amplification efficiency in low copy DNA

(3) Identification of suitable reference genes for the *in vitro* system

(4) Effect of different normalization methods on the target gene expression profile.

2. RESULTS

Details of the experimental protocols can be seen in the Chapter 2, section 2. Briefly, the human myelomonocytic cell line THP1 was differentiated by 1.2% DMSO prior to stimulation. 2 x 10^6/ml of differentiated cells were incubated with different concentrations of mycobacterial antigens (e.g. sonicated M.tb, sonicated *M. vaccae* or LPS) and cells were harvested at various time points. The concentrations of antigens used in the experiments were optimised in pilot experiments, using IL12p40 cytokine release as the readout. Student's t-test was used for the analysis of differences.

2.1. Selection of concentrations of sonicated *M. vaccae* and sonicated *M. tuberculosis* for use as stimulus in RT-PCR experiments.

It was found that the two sonicates gave different patterns of response, with different time-courses. This made selection of equivalent concentrations very difficult, and use of many different concentrations for RT-PCR time-course experiments was not feasible. The concentrations chosen caused roughly equivalent release of IL-12 p40 from THP1 cells (Figure 3.3. A) and also caused optimal increase of IL-10 mRNA in THP1 cells (B and C), though the peak occurred at 6hrs with sMtb (B) and 18hrs with sMv (C).

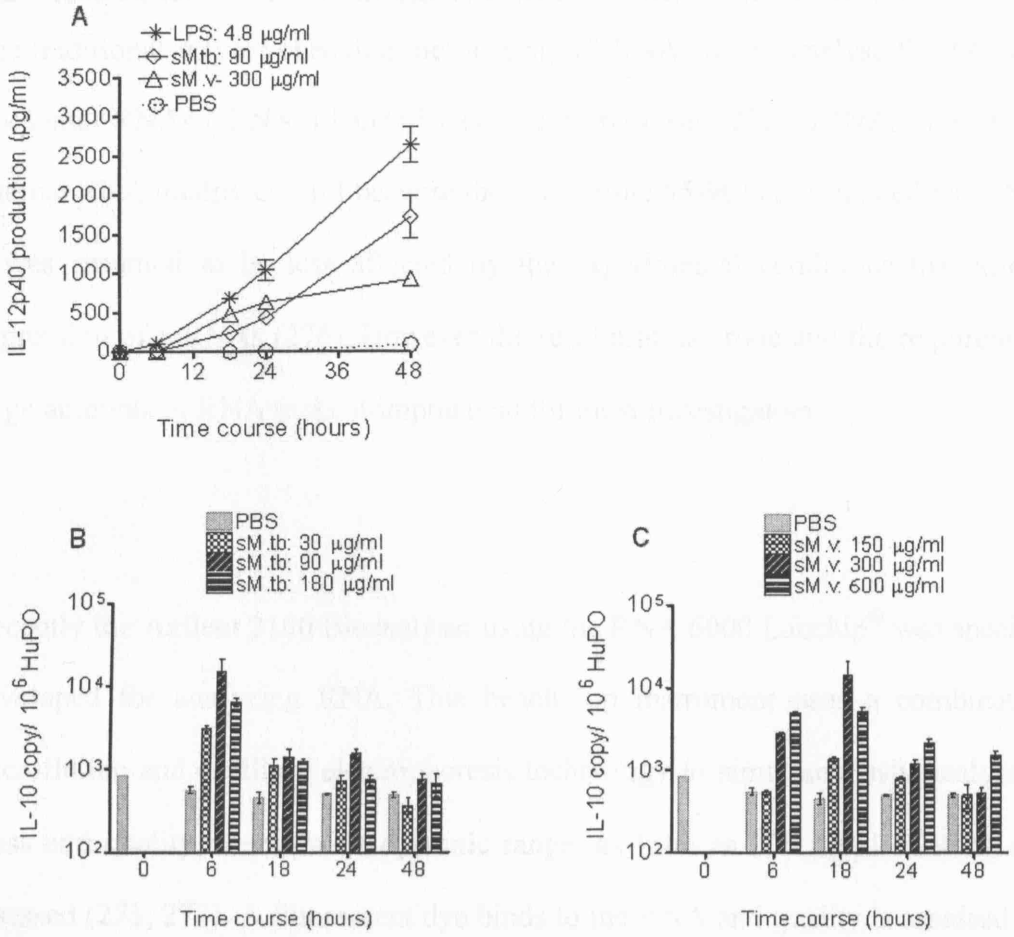


Figure 3.3. Selection of antigen concentrations of sonicated *M. tuberculosis* (sMtb) and *M. vaccae* (sMv) for use as stimuli *in vitro.* Macrophages-like THP1 cells were titrated with sMtb sMv and LPS for various period of times. Supernant protein level of IL-12p40 secreted by antigens was used to determine the optimum concentrations for each antigen.

(A). IL-12p40 protein level triggered by selected optimum concentrations of antigens.

(B) Titration of sMtb and mRNA level of IL-10. Incubation with sMtb induced mRNA encoding IL-10 as early as 6hrs. The expression level of IL-10 gradually decreased throughout the incubation period.

(C). mRNA level of IL-10 and titration of sMv over time. In contrast to sMtb, maximun induction of mRNA encoding IL-10 was found at 18hrs with the concentration of 300 µg/ml.
Results were from one of the repeat experiments and are presented as means ± SD.

2.2. Assessment of RNA integrity and concentration by Bioanalyzer

The traditional way of checking the integrity of RNA was to analyse the 18s and 28s ribosomal RNAs (rRNAs) bands by gel electrophoresis (275). rRNAs was chosen as internal RNA quality control because they constitute 85-90% of total cellular RNA and it was assumed to be less affected by the experimental conditions that affect the expression of mRNAs (276). However the resolution is crude and the requirement for large amounts of RNA make it impractical for most investigators.

Recently the Agilent 2100 Bioanalyser using the RNA 6000 Labchip® was specifically developed for analyzing RNA. This bench top instrument uses a combination of microfluidic and capillary electrophoresis technology to simultaneously analyse RNA mass and quality over a wide dynamic range; as little as 200 pg/µl of RNA can be assessed (271, 277). A fluorescent dye binds to the RNA and quality is assessed by the ratio of the area of the 18s and 28s rRNA peaks (Figure 3.4) compared to a ladder that is concurrently loaded into the disposable chip. The advantages are simultaneous assessment of RNA quality and quantity and the requirement for very small amounts of RNA.

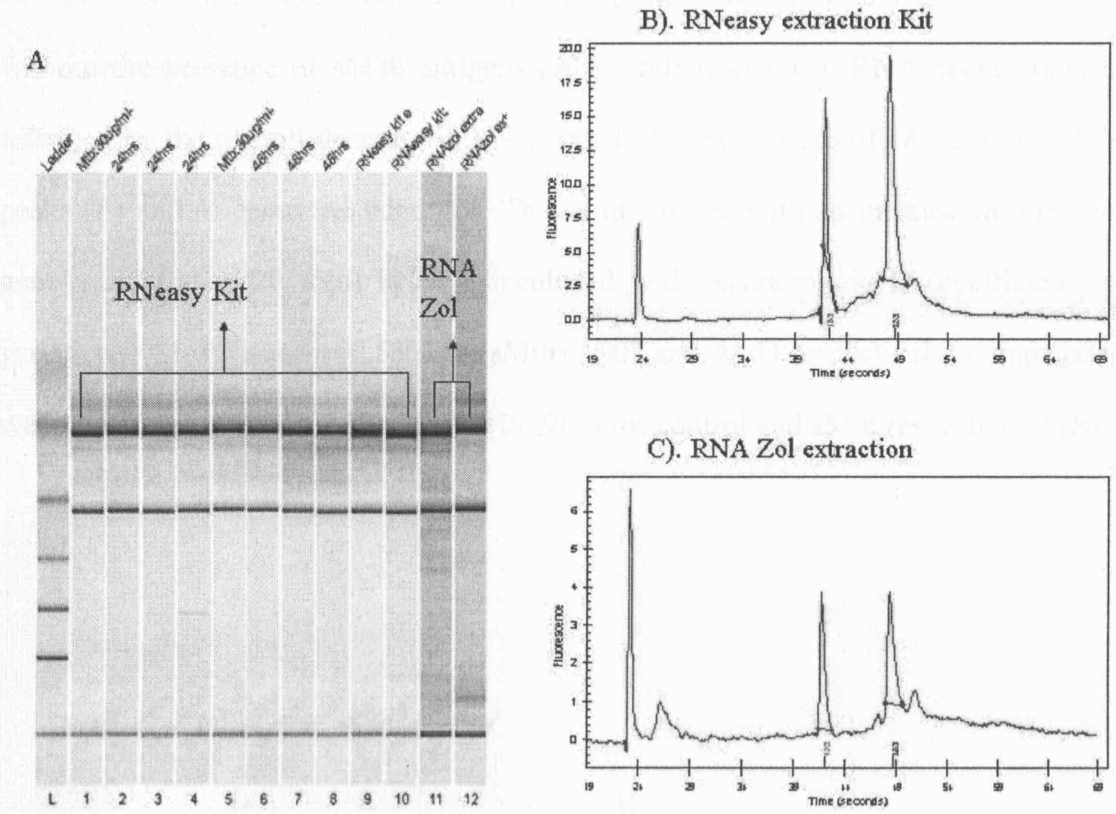
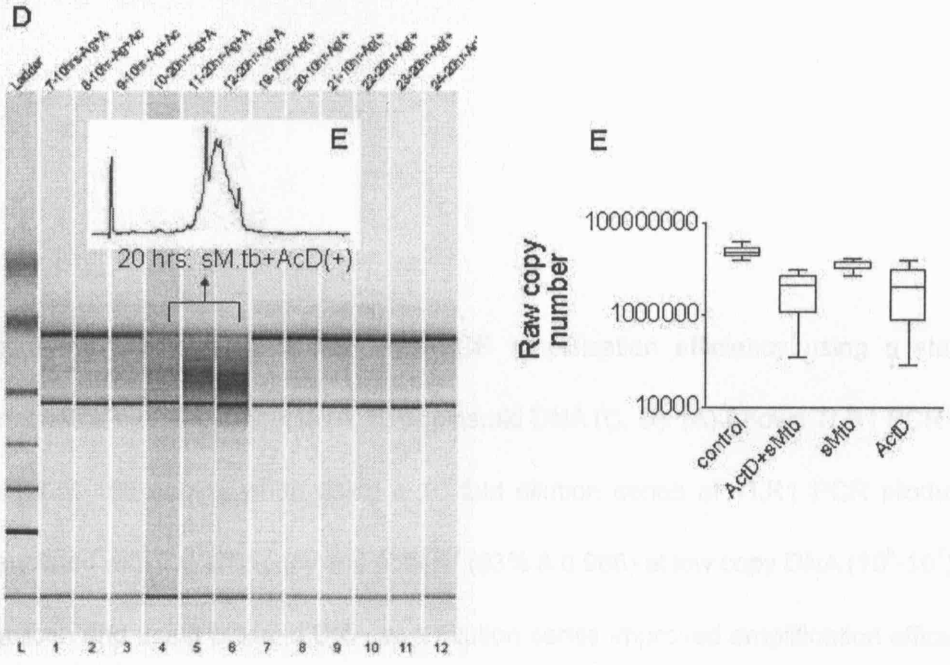


Figure 3.4. Assessment of RNA integrity using the Agilent 2100 Bioanalyser. Graph A shows a good RNA quality with no genomic DNA contamination or degraded RNA by gel electrophoresis. RNA was extracted from THP1 cells using either a commercial kit (Qiagen) or the conventional acid-guanidinum-phenol-chloroform method (278). When analysed by an electronic capillary electrophoretic tracing, sharp 18s and 28s bands against a reference RNA standard were shown (B & C). These results suggested that extraction methods had no effect on RNA quality and that incubation of THP1 cells with antigens did not alter RNA quality.

However, cells incubated with Actinomycin D (10 μg/ml) for more than 20 hrs with or without the presence of sM.tb antigens (90 μg/ml) resulted in RNA degradation, as reflected by the overall decrease in RNA signal, decreased ratio of 18s and 28s rRNA peaks (E) and smeared gel band (D). This is in parallel with an increase in reference gene variability (~20 fold) in cells incubated with Actinomycin D (coefficients of variation = 81.75% and 100.53% for sMtb+ActD and ActD respectively) compared to wells without ActD (CV= 30.81% and 26.96% for control and sMtb respectively) (E)

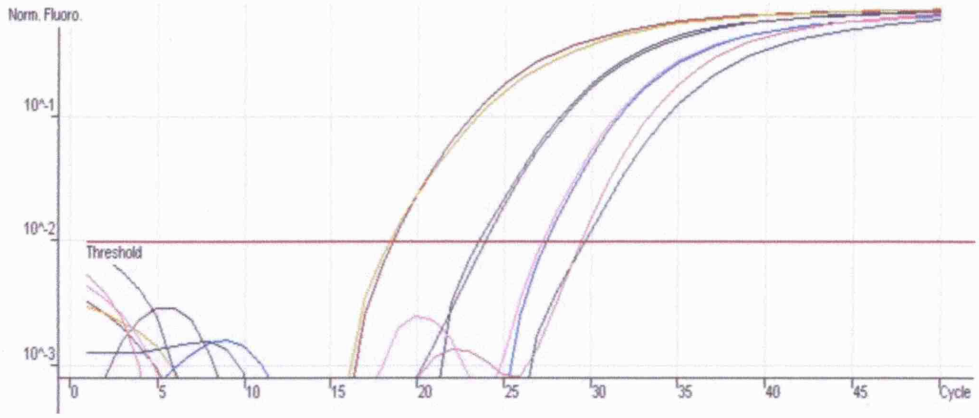


2.3. Optimization of amplification efficiency at low levels of cDNA

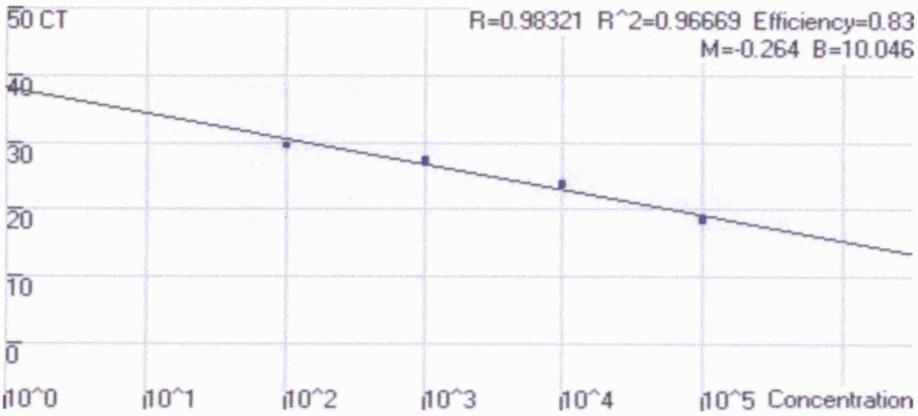
The literature suggests that some members of the TLR family have low basal expression levels as well as great donor variation (203). Therefore, we predicted that low copy measurements (~1000 copies to 10 copies or Ct<25) would be required for accurately quantifying TLR expression. Ideally the standard curve should cover the range of target gene expression because the accuracy of the absolute quantification assay is entirely dependent on the accuracy of the standards.

Figure 3.5 Comparison of TLR1 RT-PCR amplification efficiency using a standard curve constructed by PCR products (A, B) or plasmid DNA (C, D). (A) Shows TLR1 PCR amplification limited at 100 copies when using a 10 fold dilution series of TLR1 PCR product. This was associated with low efficiency and poor R^2 (83% & 0.966) at low copy DNA (10^5-10^2) (B). Cloning amplicon and using plasmid DNA as a dilution series improved amplification efficiency (C) and R^2 (D) in the low copy dilution series (83% &0.966 v.s. 100% & 0.996, respectively). But it was still limited after 26 cycles of amplification (estimated 100 copy number) (C).

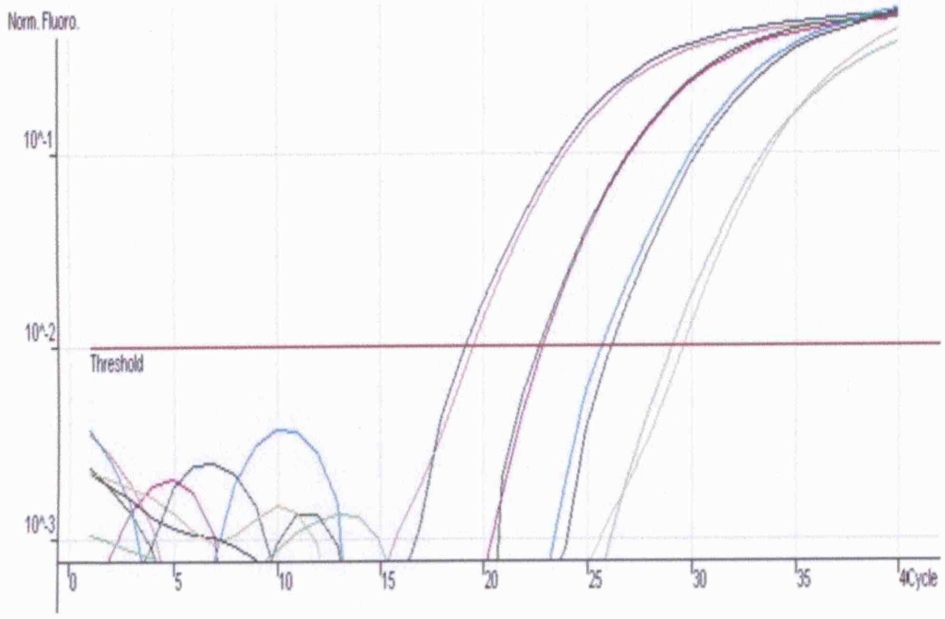
A. TLR 1: PCR dilution series: ~100,000 to ~100 copies



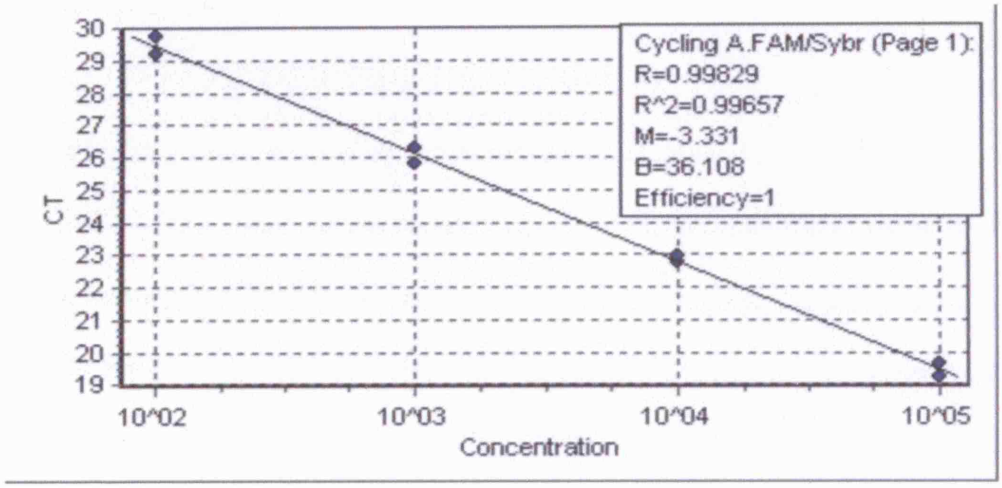
B. Amplification efficiency of TLR1 when using a 10 fold dilution series of TLR1 PCR product



C. plasmid DNA dilution series: ~100,000 to ~100 copies



D. Amplification efficiency of TLR1 when using 10 fold dilution series of plasmid DNA



2.4. Normalisation method: Total RNA and reference gene

Normalisation is necessary to control for experimental error between samples which can be introduced at a number of stages throughout the procedure. Total RNA or reference genes are commonly used. Normalisation to total cellular RNA has been shown to produce quantitative results that are biologically meaningful (279). However total RNA levels may be altered under the experimental condition (280, 281). Therefore, observed differences in gene expression between samples can simply be due to increased total RNA and not necessarily due to increased expression of the specific gene. In order to understand the effect of the various experimental conditions used in our study on the total RNA level in our system, the RNA concentration after each treatment was quantified using the Agilent Bioanalyzer. The results suggested that the total RNA concentration in THP1 cells was no different in the control (PBS) and treatment groups (sM.vaccae, sM.tb and LPS) over 48 hours of *in vitro* culture (Figure 3.6. (A))

We recently identified a suitable reference gene, Human Acidic Ribosomal Protein (HuPO or RPLPO), which varied by less than two fold in tuberculosis patients (45). To establish the most appropriate normalisation methods for these experiments, validation of suitable reference genes was carried out in the THP1 system. In addition to HuPO, two other commonly used reference genes, GAPDH and β-actin, were selected for the *in vitro* study (due to the limitations of the cost, this study was unable to assess a large panel of reference gene). Figure 3.6 (B) demonstrates that β-actin had the greatest variation (~4-5 fold) under the experimental conditions followed by GAPDH, then HuPO (coefficients of variation = 54 %, 38 % and 30 % respectively).

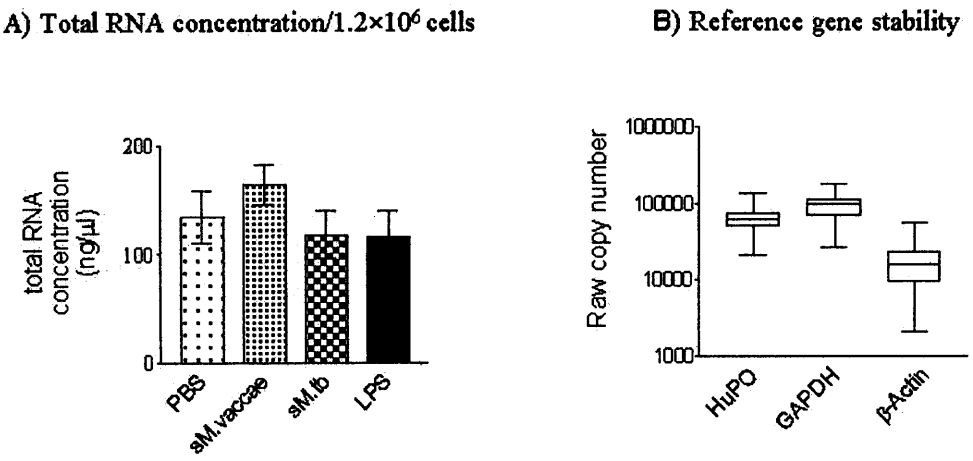


Figure 3.6. Quantification of total RNA (A) and validation of 3 potential reference genes in the THP1 model (B). Differentiated-THP1 cells were treated with 3 antigens: sonicated M. *vaccae*: 150 µg/ml sonicated Mtb: 30 µg/ml, LPS: 4.8 µg/ml and PBS as control for 6hr, 18hr, 24hr and 48 hr. 2.5 ng total RNA were used for each PCR reaction. Results in (A) are representative of two experiments and are presented as mean ±SD. Results in (B) are presented as median and 25th and 75th percentiles. Experiments were performed in triplicate wells for each antigen and each time point.

2.5. Normalization methods and TLR2 expression profile

The next question asked was whether variation in the reference genes under the experimental conditions had effects on the gene quantification results. TLR2 mRNA was normalised by four normalisation methods: total RNA (2.5 ng RNA), HuPO, GAPDH and β-actin. Figure 3.7 shows the effect of different normalisations on apparent TLR2 expression following treatment for 6 and 24 hours. When TLR2 mRNA was normalised to β-actin there were no significant differences at 6 hours between sM.tb antigen and control cells and those stimulated with *sM.vaccae* antigen (Figure 3.7.D). However when the samples were normalised to total RNA (A), HuPO (B) or GAPDH (C) antigen challenge induced a significant increase at these time points ($p<0.001$) (Figure 3.5). Conversely GAPDH (C) and β-actin (D) normalisation resulted in no significant differences at 24 hours while all other normalisation strategies indicated a change ($p<0.001$).

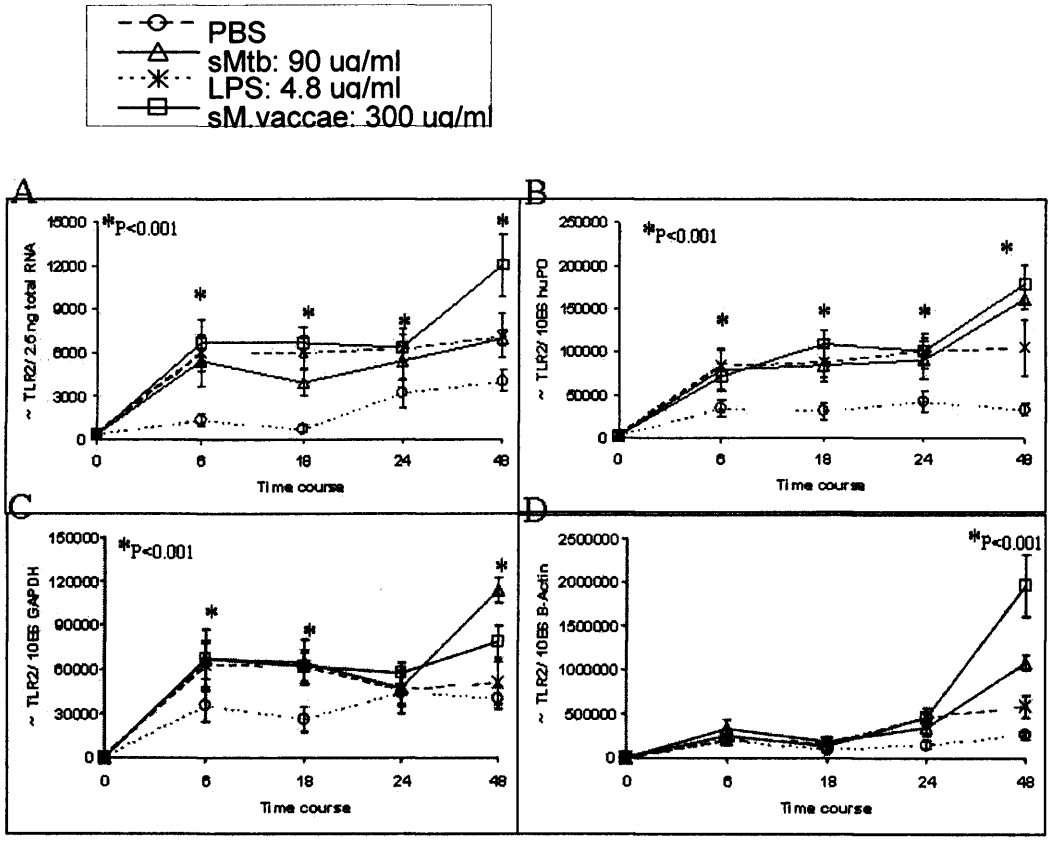


Figure 3.7.A-D shows the kinetics of TLR2 mRNA expression normalised against total RNA (A) & 3 HK genes: HuPO (B), GAPDH (C) and β-Actin (D). Total RNA and HuPO normalisation generated a similar result ($p<0.001$), whilst, β-Actin normalisation only detected a differences at the 48 hr time point ($p<0.001$). Results are from one representative experiment and are presented as means ± SD of triplicate wells.

3. DISCUSSION

Data presented in this chapter suggested that the validity of gene quantification results can be influenced by RNA quality, amplification efficiency when little template is present, and stability of the reference gene in the experimental system being studied.

3.1. Factors that affect the reproducibility of the PCR assay

Reproducibility of PCR assays is determined by the efficiency of RT, the efficiency of PCR, the quantities of target molecules and variation between operators.

- RNA quality and sample handling

The quality of the template is arguably the most important determinant for the reproducibility of PCR due to its influences in both RT and PCR steps. Inhibitors can be derived from samples themselves or exogenous contaminants (282). There are numerous components within blood and tissue that can inhibit RT or PCR efficiency, such as the presence of heme compounds, fat, glycogen, Ca2+, salt and DNA binding proteins (282, 283). Heparin in blood samples to prevent blood clotting was reported to inhibit the RT step in a dose-dependent manner when the concentration exceeded $1x10^4$ U/ml (284). Exogenous contaminants such as glove powder and phenol/alcohol derived from the extraction residues can also have an inhibiting effect in PCR assays (277).

- Reverse transcription efficiency

The RT step is the source of most of the variability in a kinetic RT-PCR experiment and for each enzyme chosen the conditions must be optimized (285, 286). Inhibitors carried over from the RNA isolation process can affect the RT efficiency (e.g. salt, phenol, alcohol) (287). Another source of variability is the choice of priming method

used to generate cDNA from the transcript molecule, which can be either target gene-specific or non-specific (286, 287). The advantage of target gene-specific priming is that the same primers can be used for both RT and PCR steps, hence allowing reduction of inter-assay variation. The target gene-specific forward primer is designed to reverse transcribe cDNA from transcript and the same reverse primer is used for the subsequent PCR assay. The disadvantage of this assay is that for each gene of interest the RT reaction is carried out separately which can result in different cDNA synthesis efficiency. Target gene unspecific primers (e.g oligo dT primer, random hexamer) allow a group of genes to be assessed from a single cDNA pool, which enables direct comparison of results between the applied assays. In the present project, cDNA was synthesised by unspecific oligo-dT primers that anneal to Poly A tails contained in most mRNA. cDNA synthesised from the same batch was used for study of gene expression in each study presented in this thesis.

- PCR efficiency

Amplicon length (<250 base pair), primer design, inhibitors and amount of DNA template are the factors affecting PCR amplification efficiency (288). The optimal amplicon length for real-time PCR is less than 100 bp because shorter amplicons amplify more efficiency than longer ones and are more tolerant of reaction conditions (289). It was reported that shorter amplicons are more likely to be denatured during the 95 degree step of the PCR allowing for the primers/probes to bind efficiently to DNA molecules (289). Moreover, short amplicons can eliminate the chance of amplification of genomic DNA. However some argue that short amplicons are more likely to select a single exon. Primer design and optimization is also the key factor for optimum PCR efficiency. Carefully designed primers can avoid non-specific amplification (e.g. primer dimers or genomic DNA). In general, the criteria are that the sequence should

have less than 20%-80% of G/C content and the primer should sit in an intron/exon boundary to avoid genomic DNA amplification (275, 288). A small amount of DNA template can inhibit PCR amplification efficiency due to the Monte Carlo effect (Discussed below).

- The Monte Carlo effect

The Monte Carlo effect describes an inherent limitation of PCR amplification from small amounts of template due to differences in amplification efficiency between individual templates (290). This phenomenon is mainly related to template concentration: the lower amount of template (normally <1000 copies) the less likely the true abundance of the template will be reflected in the amplified product (see Figure 3.3 A&B). One explanation is that primer annealing to any template during the PCR cycle is a random event. Therefore when a particular template is limited there can be random differences in amplification efficiencies depending on whether the primers were able to anneal. Assays involving low concentrations of template or low levels of target gene expression will be more likely to experience the Monte Carlo effect because the probability of primer annealing is lower. Hence, it is extremely important for low target gene study to specifically optimize amplification efficiency at low copy numbers. In our experience, cloned PCR plasmid DNA improved the Monte Carlo effect but still limited sensitivity to 100 copies when non-specific SYBR Green I was used as reporter dye (Figure 3.3. C&D).

3.2. Methods of RNA quantification

RNA quantification is important for several reasons: 1) to use as little template as possible to obtain reproducible results since most of the clinical samples are difficult to access, 2) too much or too little template can interfere with the RT and PCR efficiency, 3) standarising the same amount of starting material prior to the RT step can reduce the variability of reference gene, hence improving the reproducibility of the assay.

Traditional methods of RNA quantification are spectrophotometer and the Ribo Green assay. Estimating RNA concentration by use of absorbance of UV light at 260nm (A_{260}) with a spectrophotometer posed several drawbacks such as poor reproducibility, requirement for a large amount of RNA (5 μg/ml of nucleic acid), and inability to distinguish RNA and contaminant (e.g. DNA, protein and phenol which can absorb strongly at 260nm) (291). Although there are no published comparative studies, the RiboGreen assay is widely regarded as the gold standard for RNA quantification (275). A non fluorescent dye binds to RNA and is quantified against a rRNAs reference standard using a fluorimeter. The advantages of the RiboGgreen assay over use of a spectrophotometer are greater sensitivity, linear dynamic range (from 1 ng/ml to 1 μg/ml) and the fact that it does not bind to free nucleotides, hence eliminating false readings due to degraded RNA or contaminants (292). The Bioanalyzer is a relatively new method for RNA quantification and the advantages of the Bioanalyzer are simultaneous assessment of both RNA quality and quantity and a wide dynamic range (as little as 200 pg/μl). Results obtained using the RiboGreen assay and the Agilent Bioanalyser show good correlation except at very low RNA concentrations (Prof. S.A. Bustin, personal communication).

3.3. Normalisation method and gene expression profile

We have previously demonstrated that a number of conventionally used reference genes vary widely in mRNA expression in tuberculosis (45, 46). That conventional reference genes may not be appropriate has been shown by others in many different experimental models (272-274). In the present study, we investigated the effect of sonicated *Mycobacterium tuberculosis* on the expression of Toll like receptor 2 in the THP1 human macrophage cell line. The Toll-like receptors, which have pathogen recognition motifs, initiate host innate immune responses and facilitate adaptive immune responses (293).

To establish which of three potential reference genes fluctuated the least we assessed measures of variability (25^{th} and 75^{th} percentiles and range) for each, in the antigen driven and control wells at time points 6 and 48 hours (Figure 3.4.B) As with our previous tuberculosis experimental model (45), HuPO expression varied the least with a variation of <1.3 fold relative to total RNA. Moreover normalisation with HuPO yielded results that were very similar to normalisation with RNA (Figure 3.5), and revealed that treatment with TB antigen increased TLR2 expression at both 6 and 48 hours. In contrast normalisation with β-actin suggested that this increase had not occurred at 6 hours but did occur at 48 hours, whereas normalisation with GAPDH implied the opposite, with a significant increase at 6 hours, which had abated after 24 hours.

3.4. Effect of Actinomycin D on RNA quality

Application of Actinomycin D to block transcription activity in cells is an established method for the study of RNA stability (294). To our knowledge, the effect of ActD on RNA quality as well as on reference gene stability had never been challenged. Figure 3.4. (D&E) clearly demonstrated that cells incubated with ActD for more than 20 hrs had RNA degradation, reflected in shifting of the 28s towards the 18s peak and a decrease in rRNA ratio (E). Poor RNAs quality was associated with increases in the variability of HuPO in cells incubated with ActD after 20 hrs (~20 fold with ActD and ~2 fold in wells without ActD, respectively). Increased variability of HuPO resulted in an artefactual increase in copy numbers of the target gene due to a large multiplification factor. Based on this result, for the half life study (Chapter 5, section 2) of TLR1 and its splice variant (designated hsTLR1) RT-PCR results were normalised to total RNA.

3.5. Limitation of the assay

Work presented in this thesis assumes that normalising to ribosomal RNA using the Bioanalyser (ensuring the same amount RNA is put into the reverse transcription step) is the most favourable normalisation method (45, 46). However the assumption that mRNA concentrations follow rRNA concentrations is another dogma that is waiting to be challenged in the eukaryotes and has already been challenged in prokaryotes (295). As some infectious organisms target cellular transcription, it is highly likely that infection related studies will reveal a eukaryotic example of differences in the rRNA: mRNA ratio. This will result in rRNA quantification not being acceptable.

The accuracy in detecting low gene expression was still limited at around 100 copies (or ~Ct 26) when use SYBR Green I as reporter dye and cloned plasmid DNA to construct the calibration curve. Amplification efficiency was improved (>90%) to 10 copies (~Ct 30) with R^2 =0.99, when using a hydrolysis probe as reporter. Therefore, the use of probes for low expression genes is recommended. Cloning plasmid DNA gave better amplification efficiency over PCR products, which are also reflected in the reproducibility between assays. The limitation in the use of standard curves for estimation of PCR efficiency is that it does not control the variation in the RT stage. So, a good amplification efficiency using calibration curves does not necessarily represent a good efficiency of samples.

4. CONCLUSION

Overall, results presented in this chapter have shown that the reproducibility of the qPCR assay can be compromised by several factors, such as RNA quality, choice of the calibration curves and reference genes. Stability of the reference genes can be directly affected by the experimental system. Therefore, choice of reference genes may alter expression patterns of genes of interest, resulting in statistically significant erroneous results between groups. In conclusion, HuPO is the most stable reference gene (<1.3 fold) in our experimental system. Normalisation to total RNA and to HuPO gave similar results in gene expression, indicating suitability for normalisation. However, under certain circumstances (e.g. treating cells with Actinomycin D >20 hrs) the stability of HuPO was altered, rendering HuPO unsuitable for normalisation of such experiments.

Chapter 4. Expression profile of Toll-like receptors in patients with active pulmonary tuberculosis

1. INTRODUCTION

Immunity to *Mycobacterium tuberculosis* (Mtb) is associated with T helper 1 (Th1) activity, and involves TNF-α, IFN-γ and IL-12. Neutralisation of TNF-α leads to reactivation of disease (178), and genetic defects of the receptors for IFN-γ or IL-12 lead to increased susceptibility to mycobacteria (2). Until recently it was unclear how infected macrophages are able to link the innate immune response to subsequent T cell-mediated immunity. The discovery of the mammalian Toll Like Receptors (TLR) addresses this question (20). Ligation of TLRs triggers at least two important signals: 1) a pathway involving MyD88, one of the adaptor proteins shared by all the TLRs, leads to the activation of the transcription factor NF-κB, which governs the release of proinflammatory cytokines: 2) a second signal, which can be MyD88-dependent or -independent, drives maturation of antigen-presenting cells (APCs), and increases expression of MHC molecules, costimulatory molecules, CD40 and the chemokine receptor of CCR7. These two signals are believed to be essential for the initiation of T cell-mediated immunity (296).

Two members of the TLR family, TLR2 and TLR4, have been demonstrated to mediate Mtb-induced intracellular signalling by purified ligands or live bacilli. Several components of Gram-positive bacteria are recognised by TLR2, whilst the abundant endotoxins of Gram-negative bacteria are usually recognised by TLR4 (22). Alternative dimerisation or TLR2 with TLR1 or TLR6 allows TLR2 to recognise subtle differences in lipid configuration (23, 24). In summary, TLR2/TLR6

heterodimers recognise the lipoteichoic acid of Gram-positive bacteria, and the peptidoglycan and Soluble Tuberculosis Factors of Mtb (23, 24, 219-221, 227, 238). Agonists for TLR1/TLR2 heterodimers include the 19kD lipoprotein of Mtb, the synthetic lipopeptide of Pam3CysK4 and ara-lipoarabinomannan (araLAM) of mycobacteria (24, 43, 218, 222, 225). The 24 kDa LprG of Mtb is also a ligand for TLR 2, although it is not currently known whether TLR1or TLR6 is also involved (36).

Early work using human TLR2 or TLR4 over-expressed in cell lines suggested that viable Mtb bacilli activate cells via both TLR2 and TLR4 (297). Subsequent studies in mice with inactivated TLR genes showed that TLR2 is important in controlling (30) or surviving (32) Mtb infection. However some reports suggested that TLR4 is also important for survival (29, 253, 298) whereas others argued that the importance of TLR4 may depend on the dose of Mtb used for challenge (246) or the choice of mouse strain (241). Human studies show that polymorphisms of TLR2 or TLR4 can result in increased susceptibility to microbial infections, possibly by influencing Th1/Th2 balance (27, 28, 31, 43, 226). Interestingly a mutation in the intracellular domain of TLR2 (Arg677Trp), has been linked with lepromatous leprosy (LL) which shows a Th2-like profile (27). These patients had diminished cellular responses to both Mtb and *M. leprae* (250).

Given the critical role of TLRs in innate immunity, and in the initiation of the appropriate adaptive response, the regulation of their expression is likely to be an important determinant of the clinical outcome of Mtb infection. Moreover the presence of Th1 or Th2 cytokines can modulate the expression and also the activation level of TLR1 and TLR2 in response to *M. leprae* (43). Interestingly, Fenhalls and colleagues

reported that the pattern of TLR expression in tuberculous lung granulomas correlated with the presence or absence of immunohistologically detectable IL-4 (44). This immunohistological study is the only *ex vivo* study of TLR expression in TB of which we are aware, but in view of the increasing evidence for the importance of IL-4 (5), it raised the possibility that we can use changes in expression of TLR as markers of the immunological status of patients and their contacts. Therefore the present study used a well-validated real time PCR to document the expression profile of TLR in patients with active pulmonary tuberculosis.

The aims of this study were

- To examine the expression profile of TLR in unstimulated peripheral blood mononuclear cells as well as in cell subsets from patients with active pulmonary tuberculosis.
- To investigate expression of TLR genes at the site of infection by examining BAL samples from patients.
- To validate the findings of the clinical study by working *in vitro* with THP1 cells treated with sonicated Mtb antigens or the defined TLR2 ligand, Pam3Cysk4.

2. RESULTS

- Subjects: N=10 in each group
- Outline of patients: all patients yielded positive cultures from sputum or alveolar lavage fluid, were negative for antibodies to HIV and responded clinically to anti-TB treatment (Chapter 2, section 1.1).
- Outline of controls: these were matched to the cases for age (within 4 years), gender and ethnicity. In order to exclude latent Mtb infection in the controls, T-cell IFN-γ ELISPOT responses to ESAT-6 and CFP-10 peptide pools were determined (T SPOT TB, Oxford Immunotec, England). The details of the recruitment criteria of these subjects can be seen in the materials and methods of Chapter 2 (section 1.2).
- A reference gene was validated, and Real-time PCR efficiency was carefully optimised prior to experiment (Chapter 3, section 2).
- The optimum concentrations of antigens used in this assay were titrated in pilot studies (Chapter 3, section 2.1).
- Statistics: The Mann-Whitney U test was used for the analysis of differences in the clinical study. Time-course experiments were analysed by 2-way ANOVA with Bonferroni's correction for multiple comparisons. Analysis was conducted using GraphPad Prism and Excel.

2.1. Initial screening

During the initial screening a splice variant of TLR1, designated hsTLR1, was discovered in THP1 cells and subsequently shown to be present in peripheral blood mononuclear cells from all donors tested. Details of hsTLR1 are found in Chapter 5.

2.2. Effects of tuberculosis infection on levels of expression of TLR mRNA in whole blood

Compared to the matched controls, whole blood from patients had significantly increased levels of mRNA encoding TLR2 ($p=0.0006$), TLR1 ($p=0.004$), hsTLR1 ($p=0.0003$), TLR6 ($p<0.0001$), and TLR4 ($p=0.0002$) (Figure 4.1). By contrast, expression of TLR7 and TLR9 were not increased in patients. (TLR3, 5 and 8 were not studied).

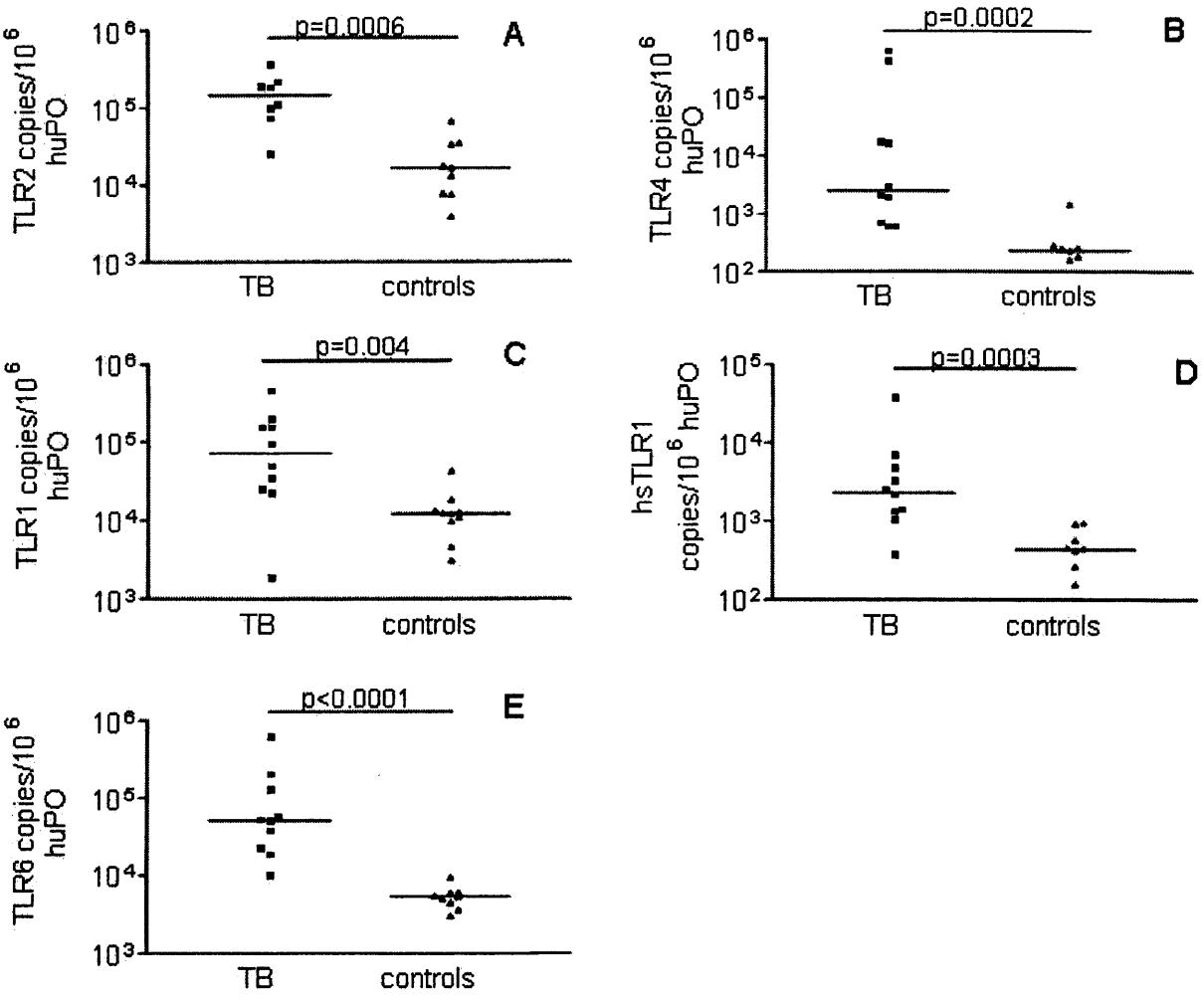


Figure 4.1. Expression of mRNA encoding TLR genes in fresh unstimulated whole blood from patients with progressive TB compared to healthy matched controls (n=10). 2.5 ml of whole blood was taken within the first 2 weeks of anti-TB treatment and transferred immediately into PAXgene Blood RNA tubes to fix the mRNA profile. Data were normalised relative to 10^6 copies of huPO. Expression of TLR2 (A) and TLR4 (B) were strikingly up-regulated in patients (p=0.0006 and p=0.0002, respectively, compared to control donors). TLR that form heterodimers with TLR2 were also upregulated in the whole blood of patients; TLR1 (C), a splice variant of TLR1 (designated hsTLR1) (D) and TLR6 (E), (p=0.004, p=0.0003 and p<0.0001, respectively).

2.3. Expression of TLR genes in cell subpopulations

cDNAs of cell subpopulations were kindly donated by Dr.K Dheda and details of cell isolation methods can be seen in Chapter 2 (section 1.4). Briefly, fresh PBMCs were separated into $CD3^-$, $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$. Purity of the fractions was confirmed by flow cytometry. Specific surface antibody staining indicated 99%, 95%, 93% and 90% purity respectively. There was an increased level of hsTLR1 mRNA in $CD3^-$ cells (mostly non-T cells) from patients ($p=0.0078$) (Figure 4.2A), and a modest increase in the $CD4^+$ population ($p=0.028$) (Figure 4.2C). Expression of TLR7 showed a small increase in the $CD3^+$ population ($p=0.043$) (Figure 4.2B).

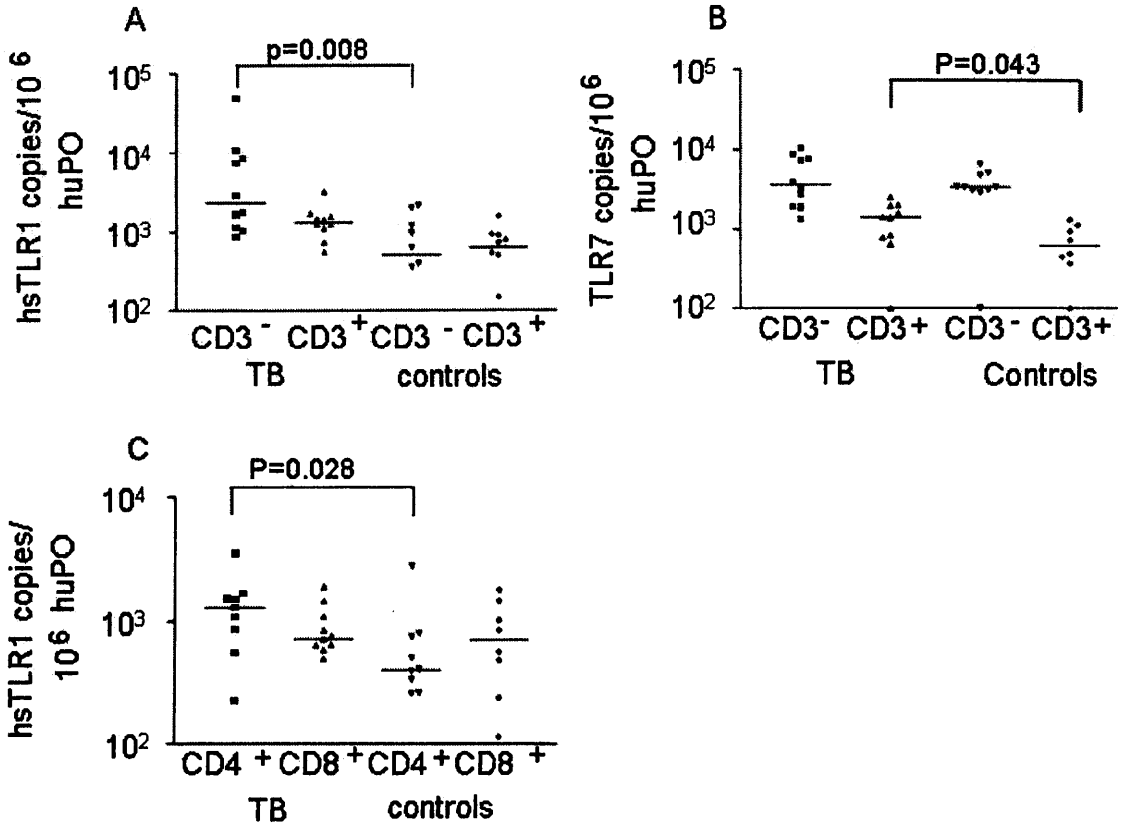


Figure 4.2. Expression of mRNA encoding TLR in cell subpopulations from fresh unstimulated whole blood from patients and controls. Fresh PBMCs were separated into $CD3^-$, $CD3^+$, $CD3^+$ $CD4^+$ and $CD3^+CD8^+$, and the purity of fractions was confirmed by flow cytometry (99% and 95%, 93% and 90% purity, respectively). There was increased mRNA for hsTLR1 in the $CD3^-$ population from patients (p=0.008) (A). Expression of TLR7 showed a small increase in the $CD3^+$ population (p=0.043) (B). The mRNA for hsTLR1 was marginally increased in the $CD3^+$ population from patients (p=0.028) (C).

2.4. Increased ratio of the splice variant, hsTLR1, relative to TLR1 and TLR6

TLR2 forms heterodimers with TLR1 and TLR6. We hypothesized that changes in the expression of hsTLR1 relative to expression of TLR1 or 6 might be relevant to the balance of different heterodimers formed. As a first step towards investigating this point, we analysed the ratio of hsTLR1 to TLR1 or TLR6 at the mRNA level.

Up-regulation of mRNA encoding of hsTLR1 in $CD3^-$ cells of patients (Figure 4.2), resulted in an increased ratio of hsTLR1 to TLR1 (p=0.017) and to TLR6 (p= 0.013) (Table 4.1). Similarly, although the increase in expression of hsTLR1 in the $CD4^+$ population of patients was small (p=0.028) this resulted in a marked increase in the ratio of hsTLR1 to TLR1 (p=0.004) and to TLR6 in this population (p=0.0031) (Table 4.1).

Table 4.1. Ratios of hsTLR1 mRNA to mRNAs for TLR1 and TLR 6 in cell subpopulations

Cell types	TLR	TB patients	Matched control	p-value
$CD3^-$	hsTLR1 to TLR1	0.6532 (0.404-0.887)	0.2745 (0.0587-0.528)	0.017
	hsTLR1 to TLR6	1.662 (0.684-3.613)	0.5645 (0.225-0.724)	0.013
$CD3^+CD4^+$	hsTLR1 to TLR1	0.9463 (0.533-1.109)	0.2213 (0.1166-0.342)	0.004
	hsTLR1 to TLR6	15160 (12360-17080)	1378 (2.454-6772)	0.0031

[a]median (25th-75th percentiles)

2.5. Expression of mRNA encoding TLR in BAL cells

In contrast to the large increases in expression of TLR in whole blood from tuberculosis patients, levels of expression in BAL from patients were not different from those seen in lavage from matched control donors (Figure 4.3).

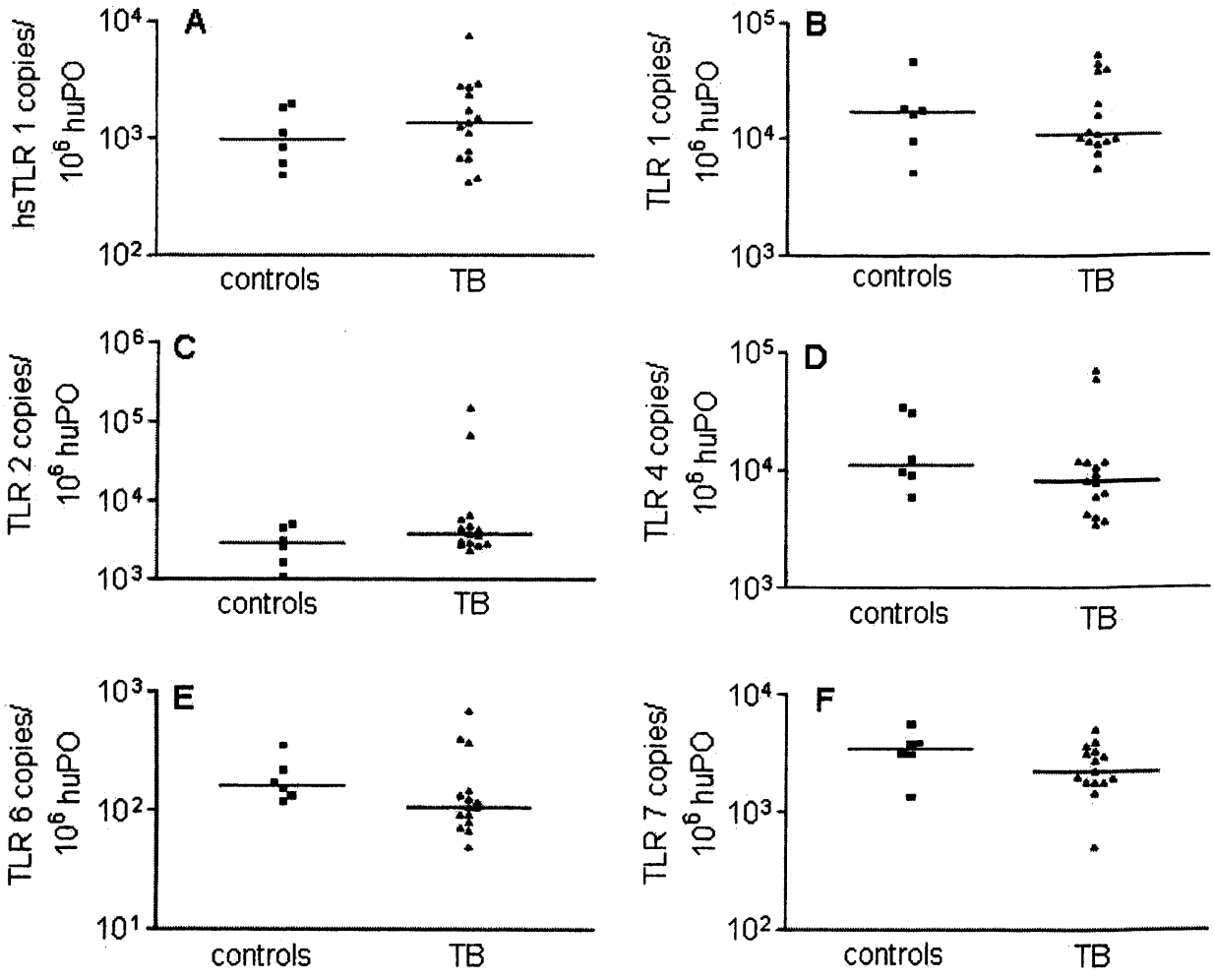


Figure 4.3. Expression of mRNA encoding TLR in BAL.

Fresh BAL cells taken from patients (n=15) and controls (n=5) were immediately placed in mRNA-stabilising buffer. mRNA levels were assayed exactly as described for whole blood. In contrast to the results with whole blood (Figure 4.1) there was no increase in TLR mRNA relative to BAL from control donors.

2.6. Effects of *M. tuberculosis* and *M. vaccae* on expression of hsTLR1 *in vitro*.

We then tested the hypothesis that the increases in TLR expression and the relative increase in hsTLR1 might be attributable to a direct effect of mycobacterial components, rather than an indirect effect requiring IFN-γ or other T cell products. Treating THP1 cells with sonicated Mtb (sMtb) (90 μg/ml) or sonicated *M. vaccae* (sMv) (300 μg/ml) increased mRNA encoding TLR1 (Figure 4.4.A) and hsTLR1 (B). A relative increase in expression of hsTLR1 led to an increased ratio of hsTLR1 to TLR1 (C) and to TLR6 (D) at 18-24 hrs, but LPS did not result in an increase in these ratios (Figure 4.4).

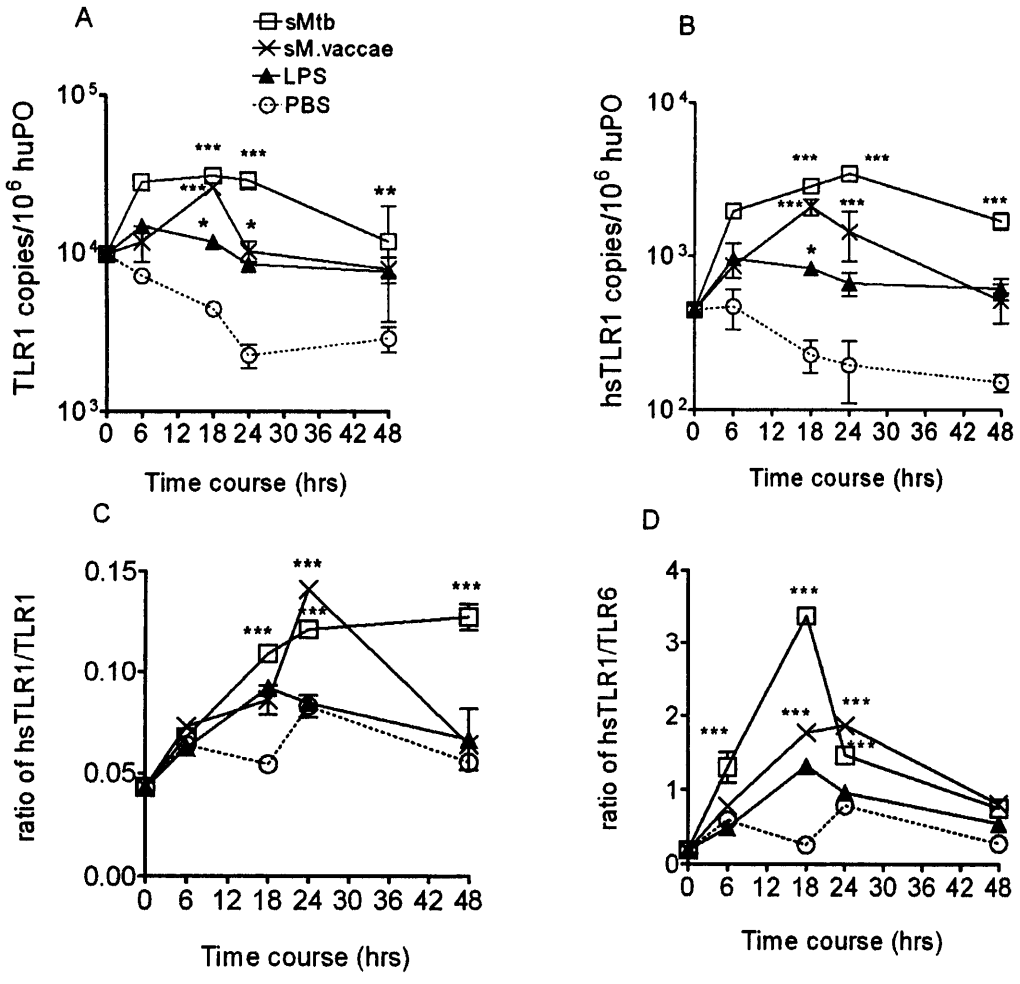


Figure 4.4. Expression of TLR1 (A) and hsTLR1 (B) in THP1 cells after incubation with

sonicated *M. tuberculosis* (sMtb: 90 μg/ml) or with sonicated *M. vaccae* (sMv: 300 μg/ml) or LPS (4.8 μg/ml). Exposure of cells to mycobacterial components resulted in up-regulation of mRNA encoding TLR1 (A) and hsTLR1 (B). However, a persistent increase in the ratio of hsTLR1/TLR1 at 48hrs was only observed in wells cultured with sMtb (C). Both sMtb and sMv, but not LPS, resulted in an early increase in ratio of hsTLR1 to TLR6 (D), ($p=0.004$, $p=0.0003$ and $p<0.0001$, respectively). Results are from one of the repeat experiments and are presented as means ± SD. 2-way ANOVA with Bonferroni's correction was employed for multiple comparisons.

2.7. Effect of the synthetic peptide, Pam3CysK4, on the expression of hsTLR1

Since mycobacteria contain components that trigger TLR2/TLR1 heterodimers, such as lipoarabinomannan and the 19kD lipoprotein of Mtb, we next tested whether a known defined trigger of TLR2, Pam3CysK4, would also increase expression of these TLR, and increase the ratio of hsTLR1 to TLR1. Incubation with Pam3CysK4 led to increased expression of both hsTLR1 (Figure 4.5A) and TLR1 (B) at 18 and 24hrs, but only TLR1 was increased at 48hrs. In the presence of sMtb the increased expression of hsTLR1 persisted at 48 hrs (A). All the stimuli, including Pam3CysK4 led to an increased ratio of hsTLR1 to TLR1 at 18 ($p < 0.01$) and 24hrs ($p < 0.01$ with sMtb; $p < 0.001$ with Pam3CysK4) but only sMtb caused a persistent increase in this ratio at 48hrs ($p < 0.001$) (Figure 4.5.C).

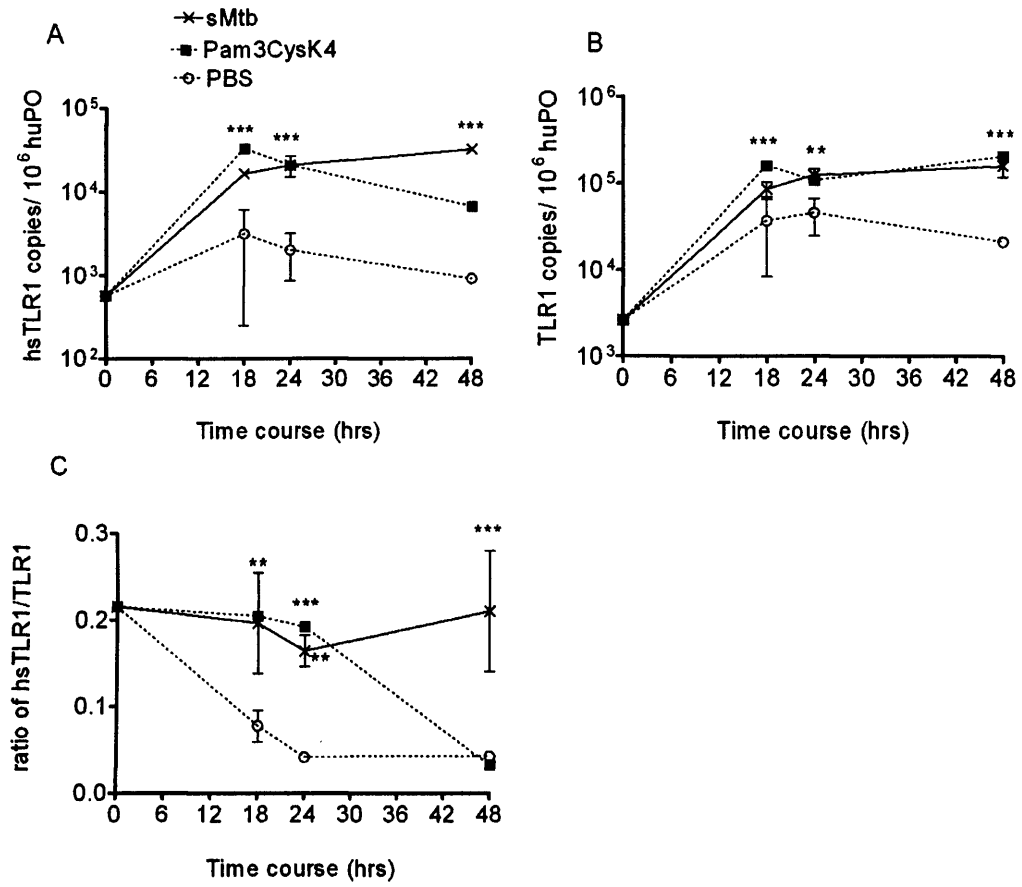


Figure 4.5. Effects of Pam3CysK4 on the kinetics of expression of mRNA encoding hsTLR1 (A) and TLR1 (B) in THP1 cells. Wells cultured with sMtb (90 μg/ml) showed increased hsTLR1 gene expression between 18hrs and 48hrs ($p<0.001$) whereas Pam3CysK4 (100 μg/ml) induced significant hsTLR1 expression at 18 and 24hrs ($p<0.001$) but not at 48hrs (A). By contrast TLR1 mRNA was increased in cells cultured with sMtb and Pam3CysK4 at all time points after 18 hours ($p<0.001$). Consequently, the persistent increase in ratio of hsTLR1/TLR1 at 48hrs was only observed in wells cultured with sMtb (C). Results are presented as means ± SEM and 2-way ANOVA with Bonferroni's correction was employed for multiple comparisons.

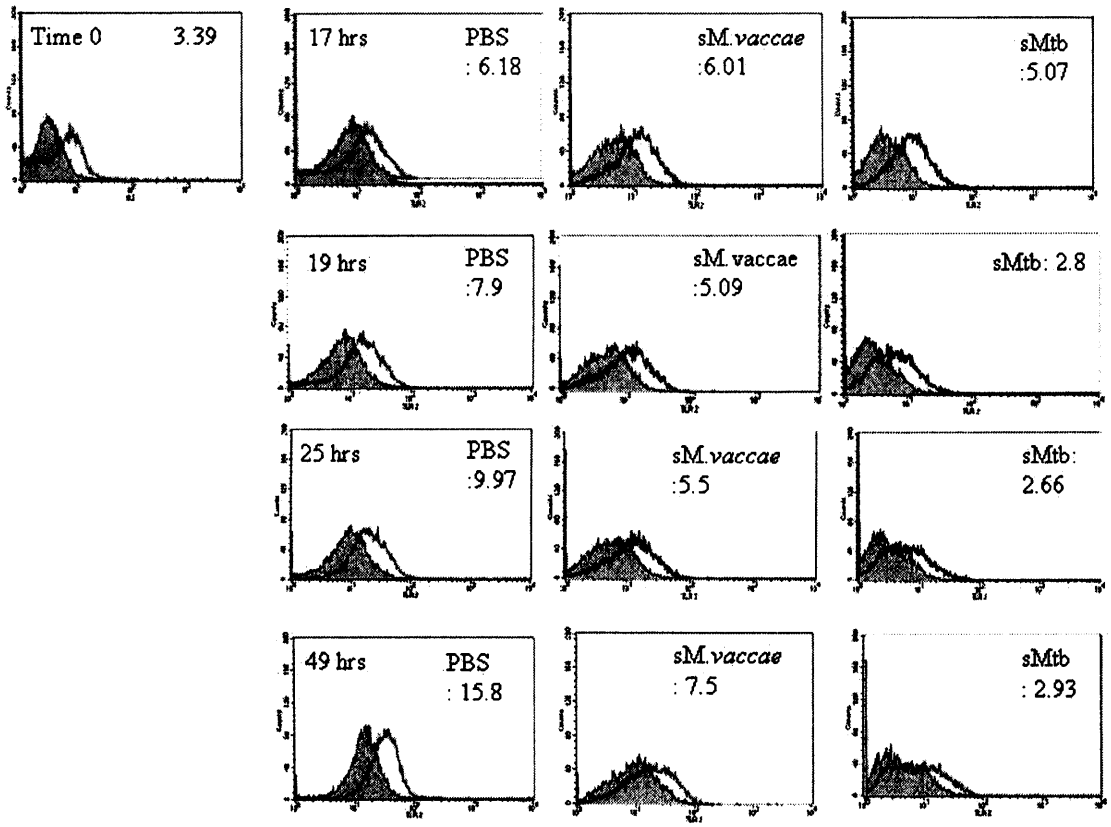
2.8. Kinetics of surface TLR2 and TLR1 expression in THP1 cells

Due to the relatively low surface expression of TLR, one colour flow cytometry was employed to measure surface protein level of TLR1 and TLR2 induced by sMtb and sMv in THP1. Antibodies directly conjugated to fluorochromes were used to label TLR1 (Phycoerythrin (PE) anti human TLR1, eBioscience) and TLR2 (FITC anti human TLR2, eBioscience) surface antigens (Details can be seen in Chapter 2, section 8). An isotype control of each antibody was also used (mouse IgG1 for TLR1 and mouse IgG2a for TLR2, eBioscience). Antibody titration was performed prior to the test. Data presented as Delta Mean Fluorescence Intensity (Delta MFI).

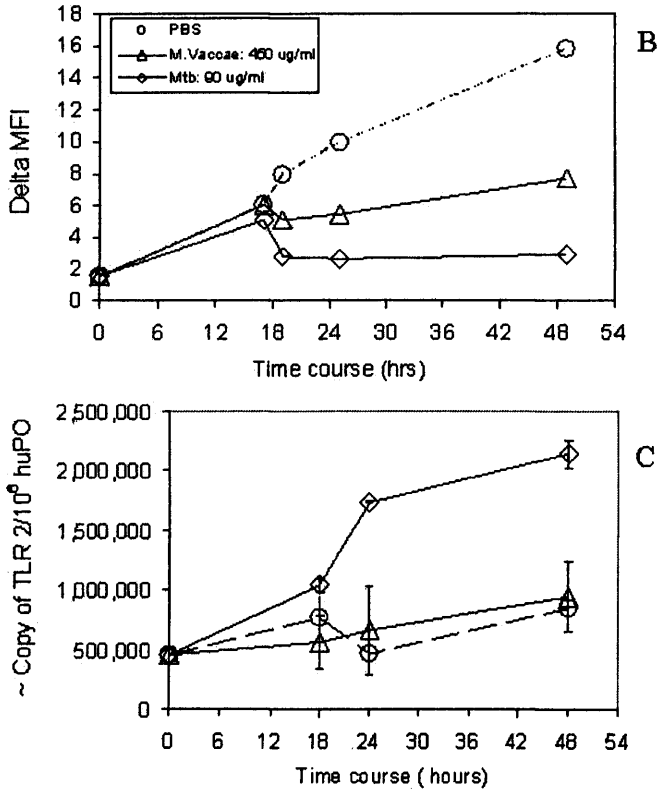
Results suggested that mRNA encoding TLR2 (Figure 4.6.C) did not correspond to the protein level (A &B) in wells incubated with sM.tb or sM.vaccae. The presence of antigens exerted inhibitory effect on surface TLR2 expression whilst long-term incubation with PBS increased TLR2 expression in macrophage-like THP1 cells. Simlarly no association was found between mRNA encoding TLR1 and TLR1 protein expression in the presence of mycobacteria antigens (data not shown).

Figure 4.6. Kinetics of TLR2 surface protein (A, B) and mRNA expression (C) in THP1 cells. THP1 cells were incubated with sMtb (90 μg/ml), sM.*vaccae* (450 μg/ml) or PBS. Cells were harvested at 0, 17, 19, 25 and 49 hrs post-treatment.

A



(A) Kinetics of surface TLR2 expression on THP1 cells in wells incubated with sMtb (90 μg/ml), sM.*vaccae* (450 μg/ml) or PBS, by one colour flow cytometry. Data are presented as Delta Mean Fluorescence Intensity (Delta MFI). Protein-specific and isotype staining were performed in single wells.



(B) Kinetics of TLR2 surface protein expression presented as Delta Mean Fluorescence Intensity (Delta MFI). Incubation with sMtb (90 µg/ml) and sM.*vaccae* (450 µg/ml) resulted in down-regulation of surface TLR2 expression compared to unstimulated wells (PBS). The Fluorescence Intensity obtained with the matched isotype control was subtracted from that obtained with the specific TLR2 antibody. Data are presented as Delta Mean Fluorescence Intensity (Delta MFI). Protein-specific and isotype staining were performed in single wells.

(C). In contrast to the down-regulation of surface TLR2 protein in the wells cultured with sMtb, 90 µg/ml sMtb up-regulated TLR2 mRNA expression at 18 hrs post-stimulation and this response increased up to 48 hrs ($p<0.01$). The results for TLR2 mRNA expression are from a representative repeat experiment presented as means ± SD.

3. DISSCUSION

3.1. General discussion

- *Ex vivo* studies that involve no *in vitro* stimuli and minimal laboratory manipulation provide a "snapshot" of the situation *in vivo*, and have the potential to suggest surrogate markers for clinical application. We have previously used this approach successfully to study the relevance of IL-4 and the inhibitory splice variant of IL-4, IL-4δ2, in TB patients and their contacts. The data presented here show that expression of mRNAs encoding TLR1, a novel splice variant of TLR1 (hsTLR1), TLR2, TLR4 and TLR6 are all strikingly increased in fresh unstimulated PBMC from TB patients, compared to their levels in cells from matched controls. By contrast, there was no such increase in expression of mRNA encoding TLR in BAL from patients. This might be explained by our previous observation that levels of IL-4 mRNA are increased more in the BAL of TB patients than in their blood. The copy number of IL-4 mRNA was almost 100x higher in the BAL from the patients studied here, compared to BAL from matched controls (6). These observations are in agreement with the only other *ex vivo* study of TLR expression in TB of which we are aware. Fenhalls and colleagues used immunohistochemical analysis of lung granulomas to show detection of TLR 1, 2, 3, 4, 5 and 9 (44), but levels of TLR2 and TLR4 were lower in those granulomas in which IL-4 mRNA was detected. It will clearly be of some interest to examine macrophages in BAL from TB patients for the patterns of activation attributable to Th1 cytokines or Th2 cytokines (so-called alternative macrophage activation) (98).

- The size of the increases in expression of TLR in peripheral blood, remote from

the clinically involved lung tissue, is surprising. This might indicate that expression of TLR1, hsTLR1, 2, 4 and 6 at the mRNA level is sensitive to increased circulating levels of proinflammatory cytokines, or to circulating mycobacterial components. This possibility was supported by the observation that infection of PBMC from normal ELISPOT negative donors with live mycobacteria could mimic some of the effects seen in the *ex vivo* studies. The finding that sMtb or sMv could cause the similar effects in THP1 cells in the absence of T cells led us to test a defined TLR2 agonist, Pam3CysK4. This molecule potently upregulated TLR2 and TLR1 and also increased the ratio of hsTLR1 to TLR1, as found in the PBMC in the *ex vivo* studies. Interestingly Pam3CysK4 replicated the effects of *M. vaccae*, but failed to cause the sustained increase in ratio of hsTLR1/TLR1 that was seen at 48 hrs in the presence of sMtb.

3.2. Possible role of hsTLR1 in immunity to tuberculosis

The strikingly increased ratio of hsTLR1 to TLR1 and TLR6 induced by the mycobacteria and by Pam3CysK is interesting. TLR1 and hsTLR1 share the same protein coding sequences. Nevertheless splice variants of non-coding regions of other genes have been implicated in the regulation of translational efficiency (299-301). Therefore a switch to hsTLR1 could modulate relative levels of TLR1 and TLR6, and so alter the composition and relative frequency of the TLR2/TLR1 or TLR2/TLR6 heterodimers. This issue was not addressed here. However there is evidence that TLR2 function is important for immunity to mycobacteria in mice and humans. Defective TLR2 activation during the infection can favour Th2 cytokines (39, 40), and could be

involved in the existence of both Th1 and Th2 cytokines at the site of infection in TB patients (6, 44). We therefore hypothesize that the outcome of TLR2 activation may depend on the ratio of hsTLR1 to TLR1 and to TLR6. Further work will be needed to explore this hypothesis.

3.3. Basal expression and distribution of TLRs in cell subsets

- $CD3^-$ and $CD3^+$

This study examined the expression of mRNA encoding TLR in fresh unstimulated peripheral blood and in cell subsets from tuberculosis patients compared with healthy contacts using a carefully validated real time PCR. Results of this study confirmed previous reports that the basal levels of TLR2, TLR4 and TLR6 are much higher in $CD3^-$ leucocytes than in T cells ($CD3^+$) whilst TLR1 and TLR7 were expressed at similar levels in $CD3^-$ and $CD3^+$ (200, 201, 203). We did not find TLR9, which according to these authors, is restricted to plasmacytoid dendritic cells (201, 203).

- $CD4^+/CD8^+$

When separating $CD3^+$ cells into $CD3^+CD4^+$ and $CD3^+CD8^+$, TLR2 and TLR4 showed a greater donor variation with only 50% of control donors expressing detectable mRNA encoding TLR2 and TLR4 (n=10). In contrast, TLR1, hsTLR1 and TLR7 were expressed at similar levels in $CD3^-$ and $CD3^+CD4^+$ and $CD3^+CD8^+$cells in controls. Interestingly, TLR6 was expressed at a detectable level in $CD3^-$ and $CD3^+$ cells but not in $CD3^+CD8^+$ and was detectable in $CD3^+CD4^+$ cells from only 4 out of 10 donors. This prompted us to speculate that TLR6 may be expressed in a small subgroup of $CD3^+$T cells such as double negative ($CD4^-CD8^-$) T cells or NK T cells. The basal

expression level of TLR1 was consistently high in both T cell subsets. The splice variant of TLR1 (hsTLR1) was also expressed in T cells but at the lower end of detection limits by Real-Time PCR. Notably, TLR1 and hsTLR1 were expressed in $CD8^+$ lymphocytes in control donors, whereas TLR6 was not present in CD8+ cells, and TLR2 and TLR4 were present in 50% and 40% respectively. Of interest, hsTLR 1 was up-regulated in $CD3^+$, $CD4^+$ and $CD8^+$ in patients even in the absence of TLR2 mRNA in some cell subsets, in particular $CD8^+$.

3.4. Effect of disease on expression of TLR mRNA in T cells

Pathogen-induced up-regulation of expression of TLRs in lymphocytes has been documented (44, 267). Fenhalls and colleagues detected TLR1, TLR2 and TLR4 in lymphocytes in tuberculous lung granuloma tissue by immunohistochemistry (44). Another study by the group of Liew looked at surface expression of TLR2 and TLR4 during activation of T cells mediated by their ligands (267). They reported that naïve $CD4^+$ T cells from cord blood expressed a low level of intracellular TLR2 and TLR4 protein, and that surface protein only became detectable after TCR activation, suggesting the trafficking of TLR2 from the cytoplasm to the cell membrane after cell activation. Conceivably, the observation of increase levels of mRNA encoding TLR2 and TLR4 in our patients may be due to an increased proportion of activated T cells or possibly memory T cells. Another explanation is the different techniques applied in the measurement of gene expression among the studies: robust Real-Time PCR versus insensitive methods of Northern Blotting and PCR. Therefore, discrepancies in the issue of TLR distribution in human T cell subsets may be due to the different techniques applied, the heterogeneity of the donors and the measurement of different activation stages of T cells.

3.5. TLR 7 and immune response

In the cell subpopulation study, a tendency towards an increase in mRNA encoding TLR7 in the $CD4^+$ population from patients was observed ($P<0.05$; Fig 4.2.B). TLR7 and TLR8 are structurally related and distributed in an intracellular compartment of endosomes (21). Their ligands includes synthetic compounds, which are used for anti-viral infection and anti-tumour treatment (210) and single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus (211-213). Notably, patients who participated in the current study were negative for HIV antibody and therefore the increase in expression of TLR7 in $CD4^+$ T cells is not due to co-infection of HIV. In fact, we also detected an increase in expression of TLR7 mRNA *in vitro* in THP1 cells. This up-regulation in mRNA encoding TLR7 was Mtb specific because LPS stimulation did not induce TLR7 expression. TLR2 and TLR7 are thought to be MyD88 dependent, but unlike TLR 2, activation of TLR 7, 8 and TLR 9 leads to the induction of IFN-α/β in a MyD-88-dependent pathway in PDCs (302). Therefore, the downstream signalling cascades triggered through TLR7 seem to differ from those triggered by TLR2. Production of certain cytokines, such as IL-10, TGF-β and IFN-α by DCs during the priming of naïve T cells has been thought to favour regulatory T cell (Treg) or helper T cell type 3 induction (119, 303, 304). Accordingly, the expression of TLR7 has been linked to the $CD45RB^{low}CD25^+$ regulatory T cell population in a murine study (206). Although the molecular mechanisms of Treg generation are currently unknown, it was believed that lack of certain TLR signals might play a part in this process. Caramalho and colleagues demonstrated that signals through TLR4 (LPS) and TLR9 (CpG DNA) inhibited the Treg generation (206). Taking into account the complexity of the immunological profile in TB patients, it will not be surprising if Treg or other types of T cell are involved in this chronic infection.

3.6. Correlation of TLR2 gene expression with the surface protein level

Data on gene expression should be confirmed at the protein level. However, flow cytometry failed to demonstrate any correlation between mRNA and surface protein level despite performing a time course experiment (Figure 4.6 & 4.7). In comparison to other pattern recognition receptors, surface expression of TLR protein is rather low. The evidence suggests that TLR are regulated at multiple stages, during transcription (305), post-transcription (306) and post-translation (268, 269). One of the explanations for the down-regulation surface TLR2 observed in this assay was that surface TLR protein was shed after binding to its ligand, resulting in less detection than on the untreated macrophages. A novel form of soluble protein of human TLR2 (sTLR2) was reported by Labeta's group (269). sTLR2 was generated by intracellular proteolytic cleavage after cell activation and was able to inhibit PBMC sensitivity to the lipopeptide of M. *tuberculosis*, suggesting a feedback mechanism controlling TLR2-mediated activation. Interestingly, they also reported that TB patients had lower levels of sTLR2 than controls but the functional relevance of this observation is unknown. Five variants of TLR2 mRNA which all contain the coding region were reported. They were generated from different usage of alternative splice acceptor (GT)/donor sites (AG) in exon II (305). Since our primers did not distinguish the normal TLR2 transcript and variants, we cannot comment on the relationship between alternative transcripts and the protein level. Exercising 5'-UTR may change the conformation of RNA secondary structure which may in turn affect the extent of TLR2 translation. Taken together, activation of TLR is regulated at multiple levels when they are triggered by pathogens. It will be of great interest to measure sTLR2 level in the supernatant of THP1 cells cultured with sMtb and also to distinguish the transcript variants of TLR2 formed in this experimental system. This may help to clarify the issue of TLR2 expression at the mRNA and protein levels.

3.7. Future experiments

3.7.1. Function of hsTLR1 in TB immunity: RNA interference (RNAi)

In order to investigate the biological meaning of the increasing ratio of hsTLR1 to TLR1 and it's effect on the function of TLR2, the mRNA encoding hsTLR1 could be knocked down using antisense or RNA interference (RNAi) in cell culture. The effect of TLR2 activation in response to M.tb antigen in the absence of hsTLR1 mRNA could be tested *in vitro*. RNA interference is a phenomenon of gene silencing at the mRNA level offering a quick way to determine the function of a gene both *in vivo* and *in vitro* (307). RNA interference (RNAi) using double-stranded small inhibitory RNAs (siRNA) exploits a mechanism that evolved to recognise and destroy double-stranded RNA from viruses. It is also involved in poorly understood housekeeping functions within normal cells. The anti-sense strand of the siRNA is used by a complex of proteins known as RISC (RNA Induced Silencing Complex) to bind to the target mRNA, which is then destroyed. In theory any gene can be knocked down in this way (308). Transfection reagents, which are solutions optimized for allowing DNA and RNA to be absorbed by cultured cells, are used to get the siRNA into the target cells. Details of procedure can be found on the website (http://www.ambion.com/techlib/tb/tb_506.html) but typically, 3-5 double-stranded siRNA molecules are designed per gene in order to find a siRNA that has a strong effect. However, high production cost limits this technology's utility, at a minimum cost of $1500 per gene.

3.7.2. Characterisation of ligands in *M. tuberculosis* causing cell activation

The increases in genes encoding TLR 2, TLR4 and TLR2 partners (TLR1, hsTLR1, TLR6) in the peripheral blood mononuclear cells of patients is not well defined in this study in terms of which mycobacterial components are inducing these changes. Future

experiments in ligand purification will help to clarify which components of M.tb induced expression of TLR. These effects are not necessarily mediated via the TLR themselves. Other PRR might be triggered by mycobacterial components, leading secondarily to upregulation of TLR mRNA.

3.7.3. *In vitro* study of activation of lavage macrophages

It will be of great interest to further investigate the mechanisms that regulated the totally different expression profile of TLR in the cellular fraction of the BAL, compared to the peripheral blood mononuclear cells of patients. Cell populations in BAL are mainly macrophages (94% in controls and 75% in patients with sarcoidosis) and a small fraction of lymphocytes (5% in controls and 24% in patients) (309). Macrophages are professional antigen presenting cells (APCs) and play an essential role in both innate and adaptive immunity.

IFN-γ-dependent activation of macrophages is a well-established feature of cellular immunity to infection with intracellular pathogens, such as *M. tuberculosis* and HIV. However, a so-called alternative activation of macrophages by Interleukin-4 (IL-4) and IL-13 exists, leading to a different type of cellular activation (98). High levels of mRNA encoding IL-4 were found in BAL cells from the same subjects in another independent study (6). Whether this IL-4 was able to down-regulate expression of TLR deserves further investigation. Macrophages isolated from lavage fluid from patients could be cultured with Mtb antigen *in vitro* and the subsequent TLR expression and activation could be measured and compared to that seen in monocyte-derived macrophages. Similarly, BAL from normal donors could be activated with sMtb in the presence and absence of IL-4, in order to see if IL-4 can stop the upregulation of TLR2 and TLR4 in these cells.

4. CONCLUSION

In conclusion, the present study indicates that the expression of several TLR genes is strikingly increased in *ex vivo* blood samples from patients with progressive TB. This increased expression was not seen in BAL samples from the same donors. This might indicate downregulation by IL-4 and anti-inflammatory cytokines at the site of disease. Much of the increased expression in blood cells may be attributable to systemic release of TLR2 agonists from Mtb and can be mimicked with Pam3CysK4 and a macrophage line *in vitro*. A novel splice variant of TLR1 (hsTLR1) was also detected, and is the subject of a separate more detailed study (Chapter 5). This splice variant showed the greatest response to Mtb in *vivo* and *in vitro*, resulting in a large increase in mRNA encoding hsTLR1 relative to mRNA encoding TLR1 and TLR6.

Chapter 5. Characterisation of the biology of hsTLR1, a novel splice variant of TLR1.

1. INTRODUCTION

TLR1 is classified as a subfamily of TLR2, together with TLR6 and TLR10, because of similarity in genomic sequence and subcellular localisation (193). TLR1 shares 68% and 48% overall amino acid identity with TLR6 and TLR10 respectively (24). Two transcript products of human TLR1, a short 3.0 kb and a long 8.0 kb transcript, were found in ovary and spleen indicating alternative splicing (310). Data from transcriptome analysis suggested that the longer product may be derived from the choice of multiple polyadenylation sites in the 3' UTR (311). Unlike the other members of TLR which are expressed at low to intermediate levels and confined to specific cell types, TLR1 is expressed ubiquitously at high levels in cells of myelomonocytic origin, polymorphonuclear leukocytes and B, T, and NK cells (200, 201, 203). TLR1 and TLR6 are normally co-expressed with TLR2 to form heterodimeric complexes (202, 218). Ligands of TLR1 and TLR6 have been defined and several lines of evidence indicate that the activation status of TLR2 can be influenced by TLR1 andTLR6, though the underlying mechanism is currently unknown (discussed in Chapter 1, section 3.4.3 and Chapter 4, section 3.2).

Chapter 4 investigated the expression profiles of TLRs in the fresh unstimulated peripheral blood and bronchoalveolar lavage cells (BAL) of patients with pulmonary tuberculosis and matched controls. During initial screening, a splice variant of TLR1, designated hsTLR1, was discovered. This splice variant showed the greatest response

to *M. tuberculosis* both *ex vivo* and *in vitro*, resulting in a changing ratio of hsTLR1 relative to TLR 1 and TLR 6. In the present study, the objective is to characterise the molecular biology of this novel molecule.

The aims of this study are:

- To characterise the cell distribution of hsTLR1.

- To examine the effect of alternative splicing of TLR1 on the RNA secondary structure by predicting RNA confirmation by Mfold

- To characterise the coding sequence of hsTLR1 by cloning a full length transcript.

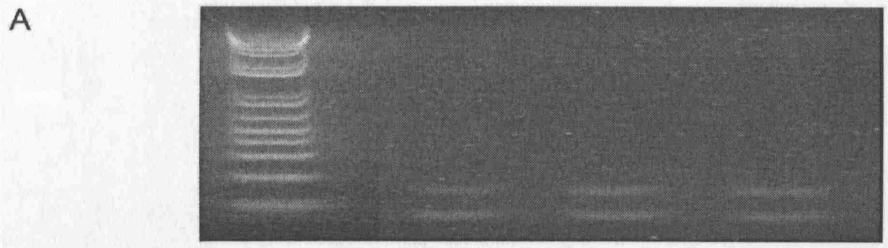
- To investigate the effect of sMtb on stability of hsTLR1 mRNA and on regulation at a posttranscriptional level.

2. RESULTS

The details of the subjects can be seen in Chapter 2 (section 1.1 & 1.2). Time-course experiments were analysed by 2-way ANOVA with Bonferroni posttests and correction. One phase exponential decay was employed to analyse RNA degradation rate. Analysis was conducted using GraphPad Prism 4. RNA secondary structure was predicted by Mfold Graphs (http://www.bioinfo.rpi.edu/~zukerm/rna/).

2.1. Discovery of a splice variant of TLR1, designated hsTLR1, in THP1 cells by real time PCR

During primer optimisation, 2 PCR products were detected using the TLR1-1-3 primers with THP1 cDNA (Fig. 5.1). Agarose gel electrophoresis suggested that the larger product was the intended amplicon (142 base pair) while the second amplicon was <100 bp (Fig. 5.1A). Sequence identification confirmed that the smaller amplicon differed by the excision of exon 2, which forms part of the 5′ UTR (Fig. 5.1B). Specific primers were designed to distinguish TLR1 from the novel the splice variant (Table), designated hsTLR1.



B

```
Query: 1   ccaaatggaacagacaagcaggcttgtcttg---------------------------- 30
           ||||||||||||||||||||||||||||||
Sbjct: 140 ccaaatggaacagacaagcaggcttgtcttgtgttaaagaaaatgagatataagtcagtta 81

Query: 31  ------------------------------------------------------------ ggtcttcatgaa 42
                                                                         ||||||||||||
Sbjct: 80  ctcccggaggcaatgctgctgttcagctcttctgtttttgtggccagggtcttcatgaa  21

Query: 43  cactaataggggtaccag 60
           ||||||||||||||||||
Sbjct: 20  cactaatangggtaccag 3
```

Figure. 5.1. Discovery of a splice variant of TLR1 in THP1 cells. Forward and reverse primers to TLR1 cDNA bind within exon 1 and 3 respectively to generate a 142 bp amplicon. (A) Agarose gel electrophoresis of RT-PCR products revealed two products: the larger molecule is the target amplicon (142 bp) and smaller molecule is less than 100 bp. (B) Sequence alignment of the two amplicons demonstrates the smaller molecule lacks exon 2.

2.2. Distribution of hsTLR1 genes in cell subpopulations

Fresh PBMCs from healthy donors were separated into $CD3^-$, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD19^+$. Purity of the fractions was confirmed by flow cytometry. hsTLR1 was expressed in all cell subsets from all donors tested (n=10 except for $CD19^+$ cells, where n=5), although the baseline expression level of hsTLR1was lower than TLR1 in control (Fig. 5.2).

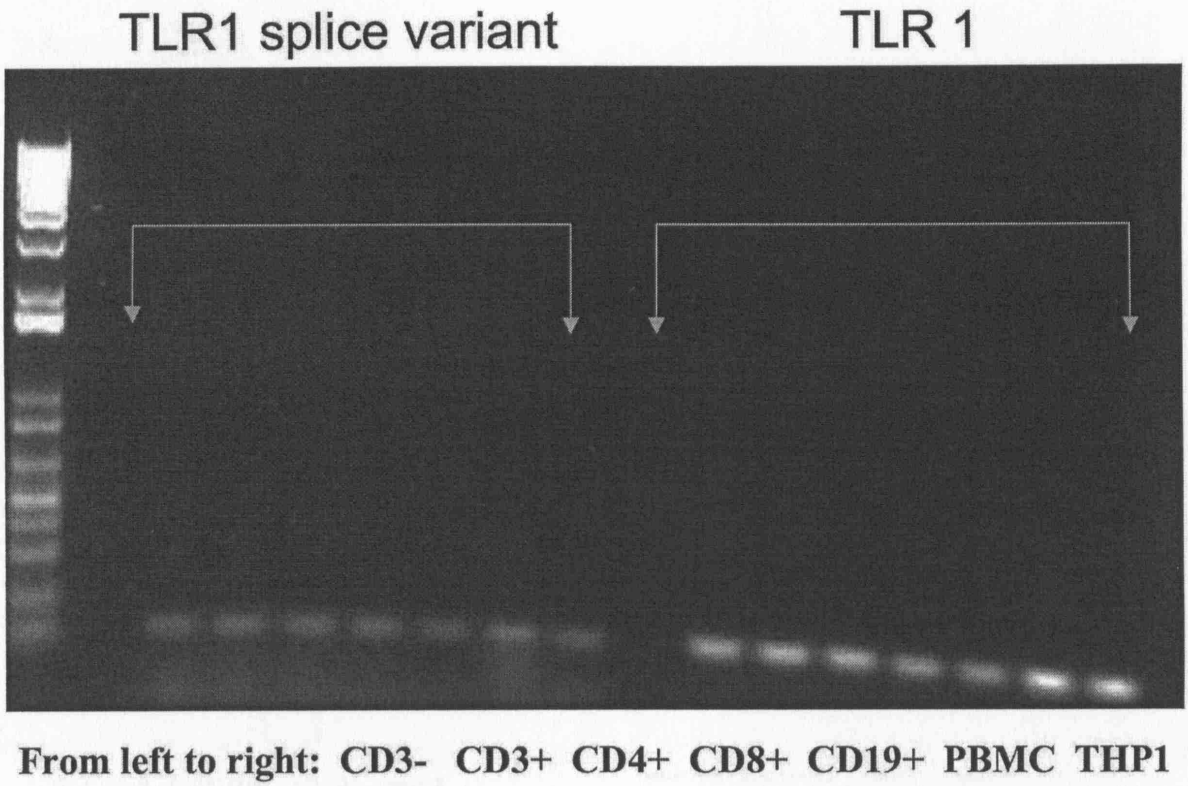


Figure 5.2. Distribution of hsTLR1 genes in cell subpopulations. hsTLR1-specific PCR using cDNA from $CD3^-$, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD19^+$ cells as template (Specific surface antibody staining by flow cytometry indicated 99%, 95%, 93%, 90% and 98% purity respectively). The splice variant was detected in all cell fractions from all donors (n=10 except for $CD19^+$ cells, where n=5).

2.3. hsTLR1 RNA secondary structure prediction by Mfold

We then investigated the effect of excision of exon 2 on the secondary structure of the RNA, as determined by Mfold using a minimum free energy prediction (http://www.bioinfo.rpi.edu/~zukerm/rna/). Exon 2 can fold upon itself. However no key structures were identified using the RNA families' database of alignments and covariance models (CMs) (http://www.sanger.ac.uk/Software/Rfam/index.shtml).

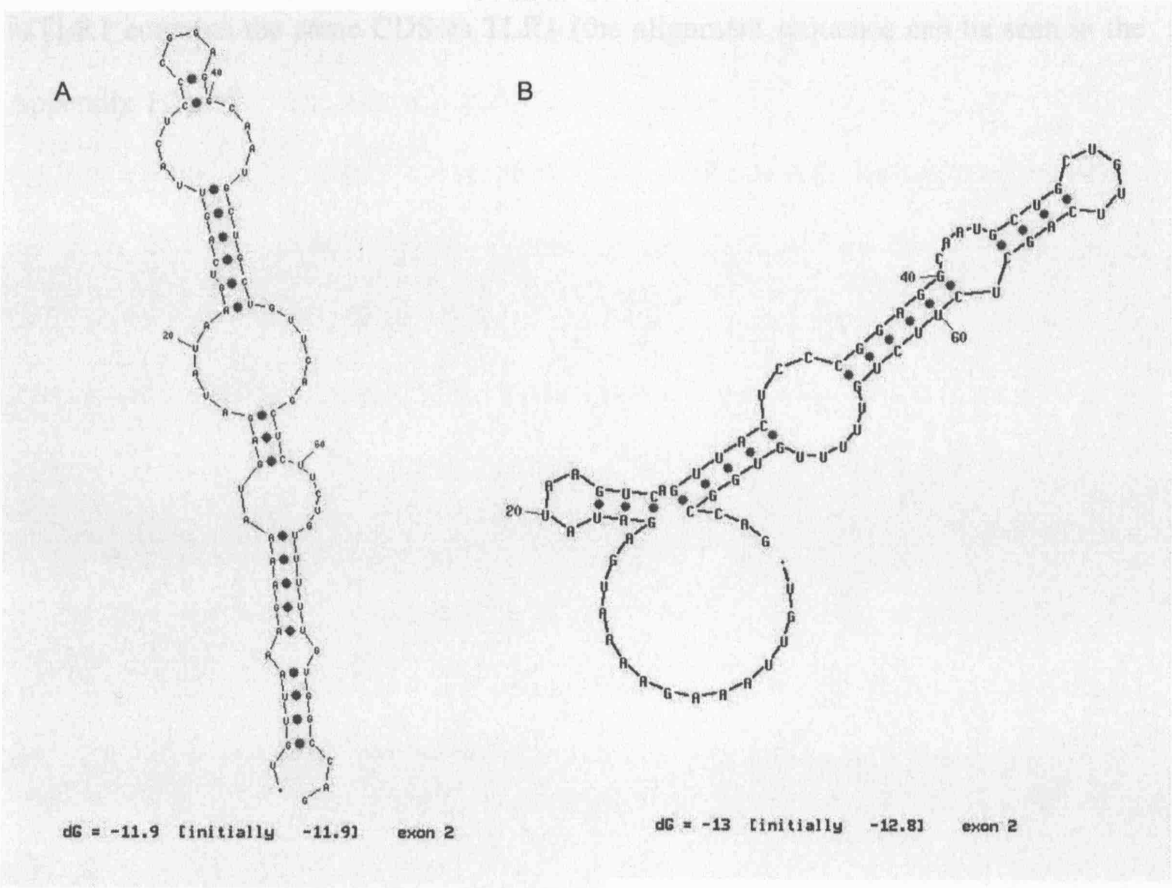


Figure 5.3. Predicted RNA secondary structure of exon 2 using a minimum free energy prediction (http://www.bioinfo.rpi.edu/~zukerm/rna/). Exon 2 can fold upon itself however there where no key structures identified using the RNA families' database of alignments and covariance models (CMs) (http://www.sanger.ac.uk/Software/Rfam/index.shtml). Two most stable predicted structures of exon 2 with calculated free energies (ΔG) of -11.91 (A) and -12.81 (B).

2.4. Full transcript cloning and sequencing of hsTLR1

Although the preliminary sequence result revealed that hsTLR1 differed in its 5' untranslated region (UTR), we aimed to determine whether the splice variant also differed in its coding sequence (CDS), entirely encoded by exon 4. The forward primer for hsTLR1 was designed to sit in the boundary of exons 1 and 3 and the reverse primer was in the 3'-UTR downstream of the CDS. Details of the primer design can be seen in Chapter 2 (section 5.3.1). Sequence analysis of this molecule confirmed that hsTLR1 contains the same CDS as TLR1 (the alignment sequence can be seen in the Appendix 1.1).

2.5. Stability of hsTLR1 mRNA and the p38 pathway

Actinomycin D was titrated and the shortened half life of c-myc mRNA (a transcriptional regulatory factor), was used as positive control for the effectiveness of Act D in the present study (262).

The increase in hsTLR1 relative to TLR1 driven by Mtb components suggested that hsTLR1 was more sensitive to Mtb than TLR1. This phenomenon could be explained either by immune cells altering their splicing ratio or by specific changes in the respective RNA half lives (½ life) when in contact with Mtb. The ½ lives of mRNAs encoding hsTLR1 and TLR1 were examined in *ex vivo* blood from TB patients (n=7) and control donors (n=4). Figure 5.4 (A) shows that the ½ lives of TLR1 and hsTLR1 mRNA were similar in whole blood from the control donors (hsTLR1 ½ life=68.07 mins vs. TLR1 ½ life=61.49 mins), but both ½ lives were prolonged in blood from TB patients. Since sMtb and Pam3CysK4 increase expression of hsTLR1 mRNA *in vitro* (Chapter 4, section 2.7) we asked whether exposure to sMtb would increase the half-life of hsTLR1 mRNA in THP1 cells. The steady-state mRNA ½ lives of hsTLR1 and TLR1 were similar in THP1 cells: 154.3 mins v.s. 180.6 mins, respectively (Figure 5.4. B). Treatment with sMtb delayed degradation of mRNA encoding hsTLR1 at 240 mins ($p < 0.001$) and 360 mins ($p < 0.001$) compared to unstimulated wells (B). sMtb-induced stabilisation in RNA encoding hsTLR1 also led to increased relative stability of hsTLR1 over TLR1 at 240 mins ($p < 0.01$).

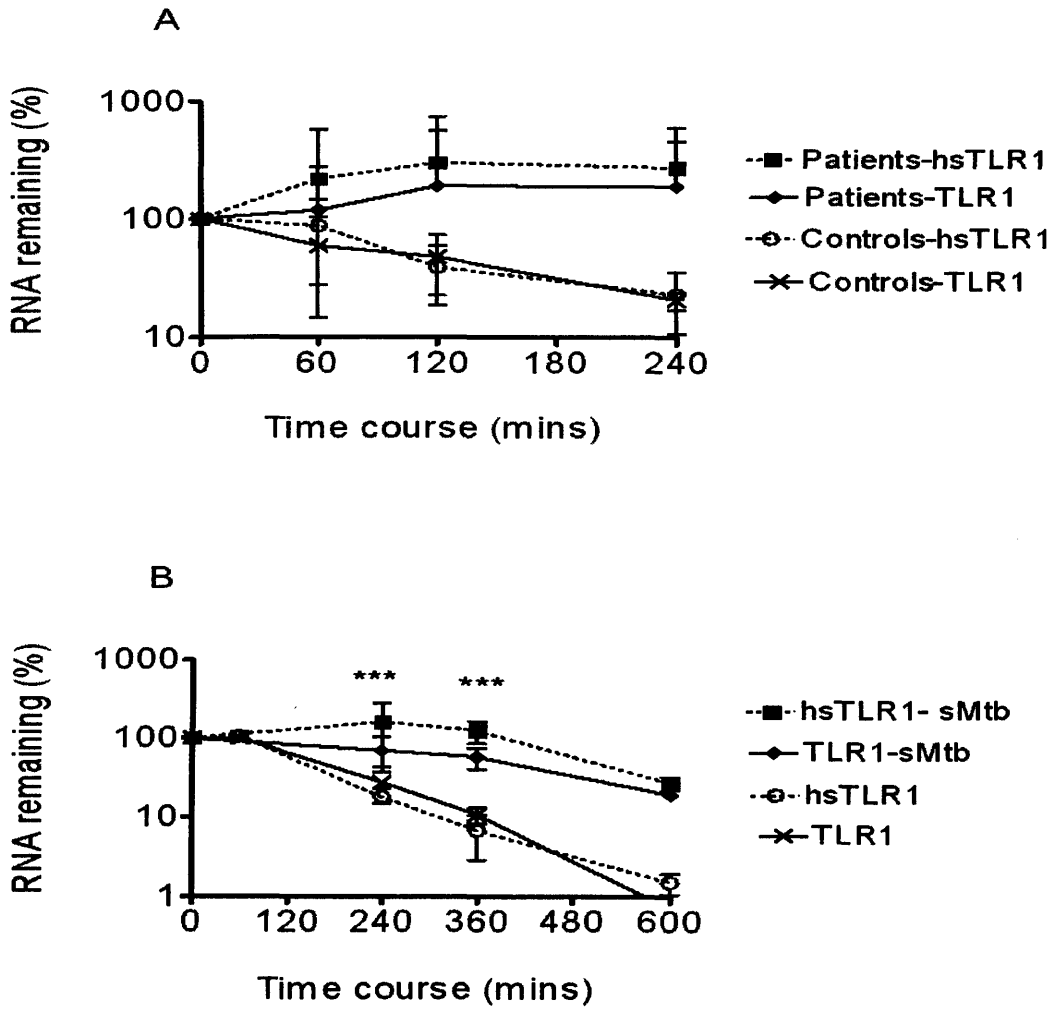


Figure 5.4. RNA stability in whole blood and THP1 cells. Whole blood cells from control donors (n=4) and TB patients (n=7) were incubated with 5 μg/ml Actinomycin D (ActD) and cells were harvested at the indicated time points. There was a trend towards prolonged stability of hsTLR1 and TLR1 mRNA in samples from patients (A). THP1 cells were cultured with or without sMtb (90 μg/ml) for 6 hours. 10 μg/ml Actinomycin D was then added to each well. Incubation with sMtb delayed degradation of mRNAs encoding TLR1 and hsTLR1 at 240 mins ($p < 0.001$) and 360 mins ($p < 0.001$) compared to unstimulated wells (B). The effect of sMtb on the stability of hsTLR1 mRNA was greater than its effect on TLR1 mRNA at 240 mins ($p < 0.01$). Results are from one of the repeat experiments and are presented as means ± SD. 2-way ANOVA with Bonferroni's correction.

Mitogen-activated protein kinase (MAPK) p38 is involved in the stabilisation of mRNAs encoding a number of inflammatory cytokines, such as TNF-α and IFN-γ (312, 313). Therefore, we examined the role of the p38 MAPK pathway in posttranscriptional regulation of TLR1 and hsTLR1 mRNA. Figure 5.5. Shows that addition of the p38 MAPK inhibitor, SB202190, caused accelerated degradation of mRNA encoding SOX-9, the ½ life of which is known to be regulated in this way, but had no effect on the stability of mRNA encoding TLR1 or hsTLR1 (263).

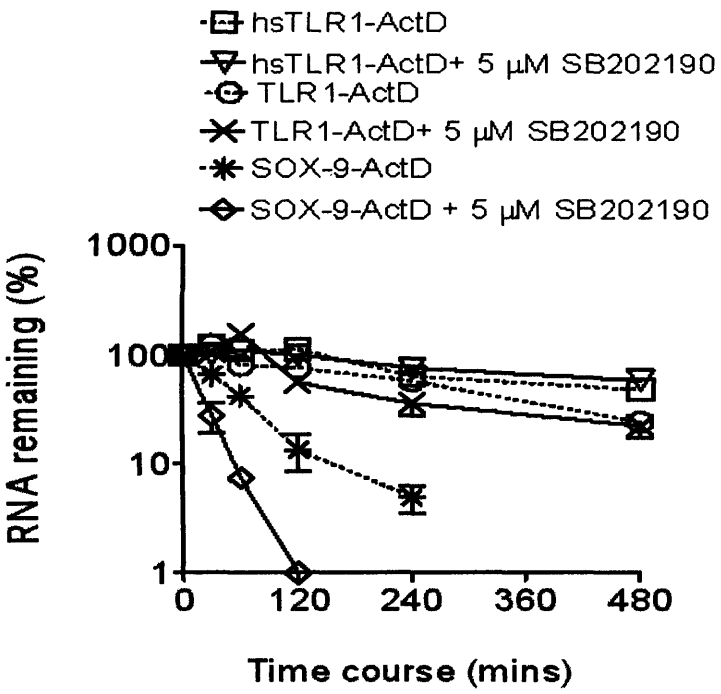


Figure 5.5. Effect of the p38 MAPK inhibitor, SB202190, on RNA stability in THP1 cells. THP1 cells were cultured with sMtb (90 μg/ml) for 6 hours. 10 μg/ml Actinomycin D was then added to each well with or without p38 antagonist (SB202190). Titration of SB202190 was performed. Results suggested that addition of the p38 antagonist had no effect on the stability of mRNA encoding TLR1 or hsTLR1. Transcription factor of osteogenic differentiation factor (SOX 9) gene expression was used as a positive control for the effects of SB202190 (263). Addition of the p38 inhibitor, SB202190, resulted in rapid SOX-9 RNA decay in THP1 cells compared to control wells (t ½= 40 mins & t ½= 107 mins respectively). Results presented as means ± SE.

3. DISCUSSION

The present study demonstrated that a novel splice variant of TLR1, designated hsTLR1 is expressed in myeloid cells, CD4+ and CD8+ T cells and B cells. The basal expression level of hsTLR1 mRNA is lower than that of TLR1 but is increased in patients with TB and also induced in THP1 cells cultured with sonicated Mtb or with the synthetic peptide, Pam3CysK. hsTLR1 lacks exon 2 which forms part of the 5'-UTR, but the coding sequence, which is fully contained in exon 4, is identical to that of TLR1. RNA prediction using Mfold suggests that the loss of exon 2 from hsTLR1 results in the absence of a stem loop structure. Stabilising RNA half life of hsTLR1 when exposed to Mtb may partly contribute to the increased ratio of hsTLR1 to TLR1 or to TLR6 as observed in the clinical study (Chapter 4), though the underlying mechanism remains unclear.

3.1. Diversity of alternative splicing of the human TLR family

A genome-wide analysis of approximately 2.1 million human mRNA and expressed sequence tag (EST) sequences revealed that around 42% of investigated genes are alternatively spliced. Many of these variants are biologically meaningful and frequently observed in the cells of immune system and nervous system (314). Transcriptome data analysis, and alignments of human transcripts deposited in the public databases predicted multiple splice sites, mainly in 5' and 3' UTR regions (311). Alternative splicing is also a feature of the plant TLR family (315). The term 'alternative mRNA splicing' is used to describe the regulated process of differential inclusion or exclusion of regions of the pre-mRNA and is an important source for protein diversity produced by same gene. Using mRNA blots to analyse tissue expression of members of TLR, Rock *et al* first reported different isoforms of TLR1,

TLR2, TLR3 and TLR4 with characteristic tissue-specificity, implying alternative splicing by the use of multiple transcription regulatory elements (310). In the case of TLR1, short 3.0 kb (designed product) and long 8.0 kb transcripts are present in ovary and spleen respectively (310). The longer product may be derived from the choice of multiple polyadenylation sites in the 3' UTR (311). A 4.0 kb TLR2 mRNA was found in the lung and a 4.4 kb transcript was observed in heart, brain and muscle (310). Alternative splicing of non a coding region of TLR2 exon II was reported, primarily confined to human blood monocytes (316).

Three alternative transcripts of human TLR4 were amplified by RT-PCR but only one transcript encoded full length protein (266). A transcript containing exons I, III, and IV yields the full TLR4 protein and the presence of exon II introduces an in-frame stop codon, which would theoretically terminate TLR4 translation after 34 amino acids resulting in a non functional TLR4. Interestingly, an additional exon between the second and third exons in mouse TLR4 was also found to contain an in-frame stop codon, resulting in a functional soluble 20 kD protein. Mutations that affect the splicing of TLR4 mRNA could favour the expression of noncoding transcripts (268). Disruptions of alternative splicing have been associated with human genetic diseases (317). It is also possible that alternative splicing events are regulated in response to developmental or physiological cues (317). As a consequence, the expression of TLR4 protein could be switched off if the second exon is included in all transcripts (Discused in detai in Chapter 8, section 2).

3.2. Alternative splicing in 5'-UTR

In many cases, alternative RNA splicing is regulated rather than constitutive and exerts biologically meaningful effects (317). The best characterised example is the regulation of the sex-lethal (Sxl) gene of Drosophila. Regulation of Sxl occurs post-transcriptionally at the level of RNA splicing (318). Genome analysis in mammals indicated that 80 % of alternative splicing events occurred in 5'-UTR (319). Variability at the 5'-ends of mRNAs may affect the protein structure if it is encoded. If it involves only the 5'-untranslated region (UTR) of mRNA, it may affect quantitative aspects of protein production at the levels of transcription initiation (320, 321), transcript stability (322) and translation efficiency (299-301). Hence alteration in 5'-UTR may provide a regulatory mechanism in controlling gene expression.

3.3. Regulation of mRNA stability

Steady state mRNA levels represent a balance between message stability and rate of gene transcription. Messenger RNAs for many molecules involved in inflammation and stress responses are normally unstable, presumably to allow rapid regulation of responses to environmental changes (323). Several components are known to influence RNA stability: the 5' cap, the 3' poly-(A) tail, adenosine- and uridine-rich elements (AREs) within the 3' UTR, sequences within the 5' UTR and sometimes motifs within the protein-coding sequence itself (294). RNA molecules can fold and base pair with themselves to form secondary structures and any changes within the structure may subsequently alter the cellular localisation, half-life and translational efficiency (294). However present data indicate that while the loss of a non-coding region of exon 2 leads to increased hsTLR1 expression in the presence of Mtb, altered mRNA half-life may may not fully explain the increased ratio of hsTLR1 to TLR1 mRNA. Both TLR1

and hsTLR1 had increased mRNA stability in the presence of sonicated Mtb in THP1 cells, and in whole blood of TB patients. In this case, post-transcriptional regulation of hsTLR1 by Mtb may provide an alternative source of RNA template for TLR1 translation or acts as antagonist of TLR1.

3.4. p38 pathway and RNA stability

Mitogen-activated protein kinase (MAPK) p38 is activated by cell stresses (e.g.heat shock) and by bacterial LPS in cells of the myeloid lineage (323). The P38 signalling pathway is a well defined posttranscriptional mechanism that stabilises genes of inflammatory cytokines, such as TNF-α, IFN-γ, IL-6 and IL-8 (312, 313, 324). Sequences containing tandem repeats of the AU-rich elements, AUUUA, in their 3'-UTR regions account for the short half life of mRNAs encoding these cytokines in the steady-state but the instability can be countered by signalling in the p38 pathway when the cell becomes activated (323). Proinfamantrory stimuli (e.g. IL-12, IL-18 and LPS) strongly activate p38 MAPK, which results in phosphorylation of several AU binding-proteins, that impede translation and reduce mRNA stability (313, 325). TLR ligands trigger cell activation and a MyD88-dependent P38 MAPK pathway has been implicated in TLR induced phagocytosis (233), bacterial killing (326, 327), DC maturation (328) as well as activation (329). It was therefore logical to investigate the possibility that the p38 pathway might mediate the Mtb-induced increased ratio of hsTLR1 to TLR1. However, our data show that, unlike SOX-9, neither TLR1 nor hsTLR1 mRNA stability appeared to be p38 MAPK dependant; treating THP1 cells with a p38 antagonist had no effect on the rate of degradation of their mRNAs (Figure 5.5). Therefore, the mechanisms modulated by Mtb to increase the stability of mRNA encoding hsTLR1 remain unclear and require further investigation.

3.5. Role of hsTLR1

The strikingly increased ratio of hsTLR1 to TLR1 and TLR6 induced by the mycobacteria and by Pam3CysK is interesting. TLR1 and hsTLR1 share the same protein coding sequences. Posttranscriptional stabilisation of hsTLR1 and TLR1 mRNA after exposure to Mtb account for increased expression in whole blood, but is not sufficient to explain the increase in mRNA encoding hsTLR1 relative to that encoding TLR1. Nevertheless the splice variant is likely to play a regulatory role. For instance, the relative levels of hsTLR1, TLR1 and TLR6 may alter the composition and relative frequency of the TLR2/TLR1 or TLR2/TLR6 heterodimers. This issue was not addressed here. However there is evidence that TLR2 function is important for immunity to mycobacteria in mice and humans. Defective TLR2 activation during the infection can favour Th2 cytokines (39, 40), and could be involved in the existence of both Th1 and Th2 cytokines at the site of infection in TB patients (6, 44). We therefore hypothesize that the outcome of TLR2 activation may depend on the ratio of hsTLR1 to TLR1 and to TLR6. Further work will be needed to explore this hypothesis.

3.6. Discussion on experimental methods

3.6.1. Methods for measuring RNA degradation rate

Determination of mRNA half life is important to our understanding of gene regulation. The most commonly used methods for measuring RNA degradation rate are to stop transcription activity by treating cells with transcription inhibitors, such as Actinomycin D (330), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (331) or α-Amanitin (332). Traditionally, results are measured by Northern blot analysis. Recent advanced in technology has provided more rapid and sensitive methods for RNA quantification, such as Real-time RT-PCR and microarray. The literature suggests that these methods are compatible with estimating the half life of an mRNA of interest. A similar result was obtained when Real-time RT-PCR was used to validate the half life of Beta-Actin that had also been estimated by northern blot in human leukemia cell lines (6.6 hrs v.s. 5.5 hrs) (333). This was also shown in a genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes estimated by microarray and selectively validated with real-time PCR or Northern blot (334). For example, human GMCSF mRNA half-life measured by microarray and Real-time PCR showed similar results in steady-state (absent v.s. 28 minutes) and activation by αCD3+αCD28 (39 mins v.s. 39 mins). RNA half-life of TNF superfamily member 14 (TNFSF14) measured by Microarray and Northern blot was 38 mins v.s. 20 mins respectively.

3.6.2. Discussion of experimental methods used in the clinical half-life study

- **Choice of sample**

Ideally, assessments of the half-life of a specific gene should conducted using a single cell type instead of whole blood. It is well known that the RNA degradation rate of specific genes is tissue/cell type-specific. Therefore working with whole blood may give rise to ambiguous results (263, 333). Whole blood, instead of a single cell type, was chosen for this study because isolation of specific cell types by FACs is time-consuming, expensive and cells may be activated during the isolation process. The PAXgene Blood RNA tube is a new commercial RNA extraction kit with the advantages of rapidly fixing the RNA profile, which was crucial for the *ex vivo* half-life study. However, there are some drawbacks related to the use of PAXgene Blood tubes. PAXgene Blood tubes are expensive and can only process 2.5 ml blood per tube. A substantial proportion of the RNA was lost during the processing, resulting in low quantities of starting material and a requirement for larger normalisation factors when analysing the data.

- **Experimental design**

The average half-time turnover for mRNA in eukaryotic cells is about 10-20 hrs and can be divided into two groups, one relatively unstable and one stable (335). Unstable genes are normally involved in regulation of cell growth, activation and apoptosis, with mRNA ½ lives ranging from around 10 min to less than 1 hour (262, 334). Cytokines, such as GMCSF, IL-4, IFN-γ, TNF-α, have a short half-time of turnover (<1 hr) for mRNA in the steady-stead but can be stabilised by growth factors, hormones or cytokines (294). On the other hand, proteins that are expressed constitutively on cell membranes such as MHC II, Fc-γ receptors and scavenger

receptors have half-lives longer than 6 hrs (336, 337). A study in murine cells cultured with LPS suggested that half-time turn-over for murine TLR4 mRNA is approximately 2.4 hrs (168 ± 32 mins), indicating a relatively high turn over for TLR mRNA compared to other PRRs (306). Based on this observation, degradation of TLR1 RNA was measured at 60 min intervals in the clinical study. However, due to difficulties in acquiring large volumes of blood sample as well as the expense of the subsequent experiment, the present assay could not be conducted in triplicate wells for every treatment and patient.

- **Data analysis**

Another difficulty encountered in half-life study was the statistical analysis. mRNA decay constants are based on the assumption that mRNA decay, like radioactive decay, is a stochastic process. Therefore, changes in mRNA concentration at any time point is a first order process, depending on the amount of mRNA present at that time. However, a biphasic RNA degradation curve occurs in some of the patients, with a rise before the fall at the end of the measurement. This phenomenon occurred in association with the presence of *M.tuberculosis* as observed in both the *ex vivo* and *in vitro* studies (Figure 5.4. A & B). The first implication of this result was that actinomycin D (ActD) failed to fully inhibit transcription activity. ActD inhibits cell proliferation by forming a stable complex with DNA and blocking the movement of RNA polymerase which interferes DNA-dependent RNA synthesis (330). The short half-life proto-onco gene c-myc was used as positive control to examine the effectiveness of ActD in the present study (262). Under the same conditions, transcription of the c-myc gene was inhibited. Furthermore, the biphasic RNA degradation curve was only observed in patients but not in control donors of which RNA appears to undergo linear degradation following addition of

ActD. It is currently unknown what causes this phenomenon. Fitzgerald *et al* reported observing the same biphasic RNA degradation phenomenon in a study examining the effect of LPS on scavenger receptor A (SR-A) gene expression in mouse macrophages (337). Authors reported increasing levels of SR-A transcripts at 6 to 8 hrs after ActD treatment in cells stimulated with LPS but no such phenomenon was observed in control wells. They concluded that this phenomenon may be due to post-transcriptional mRNA processing.

Taken together, the present study was unable to estimate half-time turn over for mRNA of TLR1 and hsTLR1 in some patients due to the few data points and the bimodal curve.

3.7. Future experiments

3.7.1. RNA transcription activity measurement

Assessing mRNA levels in the nuclear or cytoplasmic compartment allows distinction of mature versus immature RNA. The target gene level in the nucleus gives an idea of the transcriptional initiation events that occur within a cell of interest. Nuclear run-on assay is well suited for this purpose. However, isoforms of TLR1 may be generated by the same transcription start sites. Furthermore, a synthesized transcript may not be of functional relevance and can be subject to further control mechanisms such as RNA processing and transportation. Alternatively, the mRNA level in cytoplasm can be quantitated (331).

3.7.2. Analysis of the 5'-flanking region of TLR1: Investigation of promoter utilisation

In order to define underlying mechanisms responsible for the selective induction of mRNA encoding hsTLR1, a study in promoter utilisation is required. Promoters are in general responsible for the determination of the transcription start sites and influence the rate of transcription initiation (338). Use of alternative promoters has been implicated in 5'-UTR alternative transcript production (338). Analysis of the 5'-region of human RFC1 (reduced folate carrier) genes revealed that 5'-isoforms resulted from the use of multiple transcriptions start sites which were controlled by the multiple promoters (339). This is in line with much evidence indicating the role of multiple promoter utilisation in the control of alternative transcripts (340-342). Cloning the 5'-flanking region of human TLR1, starting upstream of the transcription start site from human genomic DNA should enable determination of the promoter utilisation of TLR1. This allows determination of regulatory sequences essential for initiation of transcription in eukaryotes, such as promoters (TATA-box, CCAAT-box and CpG Islands), the proximal promoter region (~20-200bp upstream) and enhancers (~10 to 50 kb upstream of the transcription start site or down-stream). Initiation of transcription activity can also be influenced by transcription factors, which interact with specific regulatory sequences and position RNA polymerase II within transcript. It is possible that the effect of *M.tuberculosis* on TLR1 isoforms may regulated by transcription factors.

3.7.3. Assessment of translation efficiency

RNA secondary structure prediction revealed that excising exon 2 resulted in removal of a hairpin loop near the translation start site in hsTLR1. Theoretically this alteration

can enhance the efficiency of TLR1 protein translation resulting in a quicker host response against *M. tuberculosis.* Several lines of evidence indicate that alternate 5'-UTRs are highly suggestive of translational control (299-301). In order to investigate this possibility, TLR1 and hsTLR1 transcripts can be subcloned into vectors containing a luciferase reporter gene, and used to transfect a suitable cell line. Translation efficiency of hsTLR1 and TLR1 could then be assessed by monitoring luciferase activity.

4. CONCLUSION

In conclusion, infection with *M. tuberculosis* increased the stability of mRNA for TLR1 as well as for hsTLR1. The mechanism of this post-transcriptional regulation is not known and was p38 pathway-independent. Although the increased half-life of hsTLR1 mRNA contributed marginally to the increasing ratio of hsTLR1 to TLR1, other regulatory mechanisms must also exist that regulate this splicing event. Future work is required to determine the functional relevance of the splice variant of TLR1 and mechanisms that modulate RNA stability in TB and in other infectious diseases. It is clear that the use of PCR primers that distinguish between the two forms is important, so as to avoid generating misleading data and missing new insights.

Chapter 6. Expression of IL-4 mRNA in peripheral blood mononuclear cells in relation to expression of TLR2

1. INTRODUCTION

When triggered by appropriate ligands TLR drive rapid cell activation, maturation and cytokine release. This response plays a role in the initial innate response to infecting organisms, but also helps to determine the nature of the subsequently generated specific immune response. In general the view has been that a Th2 response, involving generation of T cells that secrete IL-4, IL-5 and IL-13, is the default, while responses generated in the presence of TLR agonists were more likely to be Th1-biased, and associated with IFN-γ production because of the tendency for TLR to drive IL-12 release (42). Recently it has become clear that the situation is more complex. TLR4 is required for optimal development of a Th2 response (37). Moreover endotoxin, which triggers TLR4, can drive either a Th2 or a Th1 response depending on the dose used (38).

The most clear-cut examples of TLR driving Th2 responses have come from the study of TLR2. This TLR forms heterodimers with TLR6 or TLR1 and these heterodimers have different PAMP specificities (24, 224). The synthetic TLR ligand Pam3Cys is recognised by TLR2/TLR1 heterodimers. When mice are immunised with ovalbumin mixed with Pam3Cys they develop enhanced Th2 responses and increased allergic airway constriction following subsequent intransal challenge (39). Moreover murine bone-marrow-derived dendritic cells incubated with Pam3Cys expressed increased levels of IL-13 but little IL-12, IL-18 or IL-27 (39). This seems relevant to man

because human monocyte-derived DC incubated with Pam3Cys failed to release IL-12 p70, and allogeneic T cells cultured with them developed a Th2 bias (40).

The objective of this study was to examine the correlation between expression profile of TLR and Th1/Th2 cytokine in peripheral blood of patients and control donors.

2. RESULTS

The subjects had been bled as controls for a study of tuberculosis (TB) patients, to whom they were matched for age, gender and ethnicity (Chapter 2, section 1.2). They were all people who had been exposed to TB, but who did not have latent disease. In order to exclude latent TB infection T-cell IFN-γ ELISPOT responses to ESAT-6 and CFP-10 peptide pools were determined (T SPOT TB, Oxford Immunotec, England). All the donors were negative by this test. The Mann-Whitney U test and Spearman's non-parametric rank sum correlation were used; analysis was conducted using GraphPad prism and Excel.

2.1. Relationships between expression of TLR2, TLR1/TLR2 ratios and IL-4 or IL-4δ2 in fresh unstimulated whole blood from normal donors

While analysing the control group from the clinical study (Chapter 4) a correlation was observed between mRNA for IL-4 and that encoding TLR2 in fresh unstimulated whole blood (Figure 6.1A). Since the function of TLR2 is modified by its heterodimerisation partners TLR1 and 6, the question was then asked whether expression of IL-4 mRNA was also correlated to the ratio of these partners to TLR2. Expression of IL-4 was highest in PBMC with the lowest ratio of TLR1 to TLR2 (Figure 6.1B; $p=0.0007$; Spearman's rank sum correlation). The same was true for the ratio of TLR6/TLR2. Since IL-4δ2 is an antagonist of IL-4, we performed the same calculation with the values for IL-4δ2 mRNA, and found the reverse effect. The expression of IL-4δ2 mRNA is most strikingly increased when the ratio of TLR1 to TLR2 is high (Figure 6.1C; $p=0.001$).

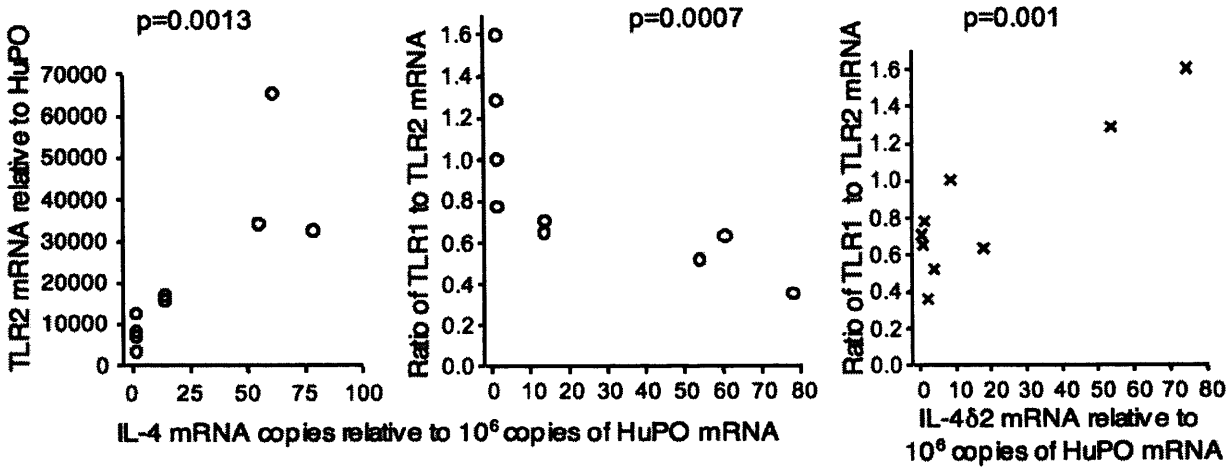


Figure 6.1.Relationships between expression of TLR2, TLR1/TLR2 ratios and IL-4 or IL-4□2 in fresh unstimulated peripheral blood from normal donors.

A) Correlation between TLR2 mRNA and IL-4 mRNA expressed per 10^6 copies of HuPO mRNA (p=0.0013).

B) Correlation between the ratio of TLR1/TLR2 mRNA, and IL-4 mRNA (p=0.0007).

C) Correlation between the ratio of TLR1/TLR2 mRNA and IL-4δ2 mRNA. The correlation seen using mRNA encoding the antagonist (IL-4δ2) was the reverse of that seen with the mRNA encoding the agonist (p=0.001).

2.2. Relationships between expression of TLR2 and IL-4 in T cells from normal donors.

The question was asked whether these relationships were due to the $CD3^+$ or the $CD3^-$ subpopulations of these normal donors. No correlation was found in the $CD3^-$ cells. However the $CD3^+$ cells of 5 donors expressed TLR2, whereas TLR2 mRNA was undetectable in cells from the other 5 donors. We therefore segregated the donors

into $TLR2^+$ and $TLR2^-$ and used the Mann Whitney U test to compare expression of mRNA for IL-4, TLR1 and IFN-γ in the two subgroups. As shown in Figure 6.2, the expression of IL-4 was mostly in the $TLR2^+$ $CD3^+$ cells (p=0.007), whereas expression of TLR1 was mostly in the $TLR2^-$ $CD3^+$ cells (p=0.015), which also tended to express more IFN-γ (ns).

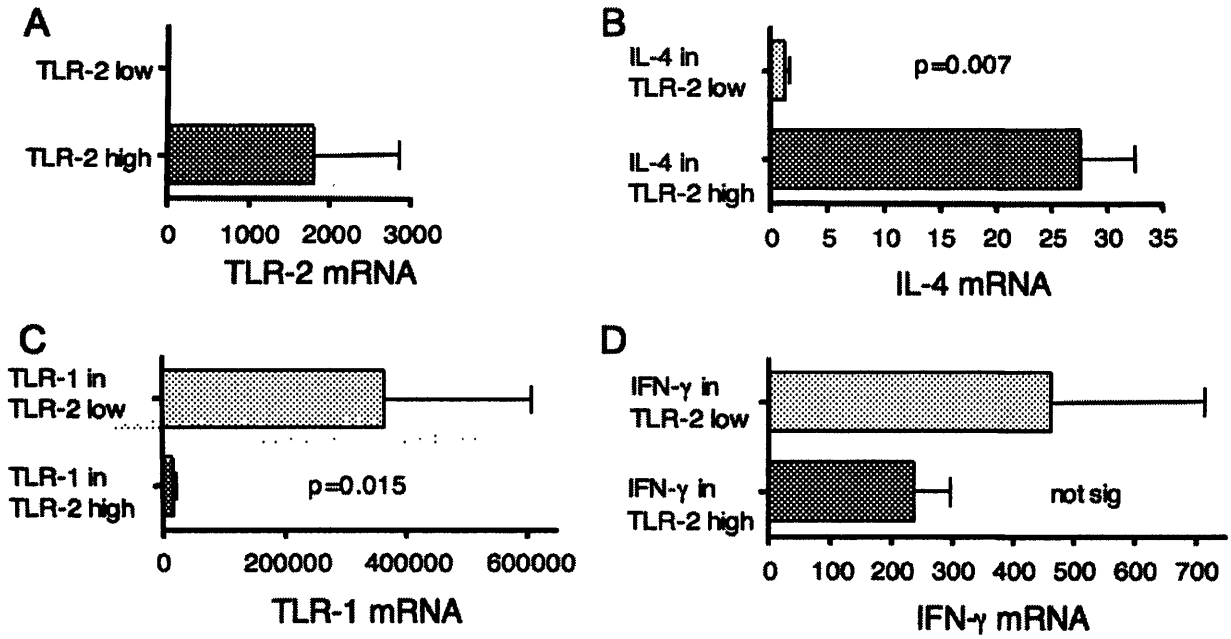


Figure 6.2. Expression of TLR1, IL-4 and IFN-γ mRNA expressed per 10^6 copies of HuPO mRNA in CD3+ cells from donors whose CD3+ cells did (n=5), or did not (n=5), express detectable TLR2 mRNA. Expression of IL-4 mRNA was confined to the TLR2 high cell populations, while expression of TLR1 was significantly higher in the TLR2 negative populations, which also tended to express more IFN-γ.

2.3. Relationships between expression of TLR2 and IL-4 in cells from patients

Finally it was investigated whether this relationship between TLR2 and IL-4 was also present in the cells from the TB patients. No such correlations were found in whole blood or their subpopulations when these were taken from patients.

3. DISCUSSION

3.1. General discussion

Clearly these results are based on a single study of a small group of normal donors, and have been determined by RT-PCR only. Therefore the biological meaningfulness of these results must be questioned. Nevertheless the statistical analysis is so strong and the results so inherently compatible with other published data, that the observations were thought worthy of comment.

The role of TLR2 is clearly complex. It has been associated with driving Th1 responses (343), regulatory T cell responses (344), and most recently, Th2 responses (39, 40). The TLR2/TLR1 agonist, Pam3Cys has been shown to act as a Th2 adjuvant both in a mouse model of allergy (39), and in human monocyte-derived DC *in vitro* (40). The present study showed that IL-4 mRNA in fresh unstimulated whole blood from healthy donors correlates with TLR2 mRNA levels. By contrast, IL-4 was undetectable in the whole blood of those donors with high ratios of TLR1 to TLR2 or of TLR6 to TLR2.

At the T cell level, IL-4 mRNA was present only in cell populations that expressed readily detectable TLR2, and as had emerged from the whole blood, a low ratio of TLR1 to TLR2. The absence of IL-4 mRNA from T cell populations that did not express TLR2 was not due to technical issues since these same TLR2-negative, IL-4-negative T cell populations expressed significantly more TLR1 (and somewhat more IFN-γ)

It is important to note that we would not have made these observations if we had not been using primers that distinguish between mRNA encoding IL-4 and mRNA encoding the splice variant, IL-4δ2, an inhibitor of IL-4 (345, 346). This cytokine behaves like a Th1 cytokine, and for instance, is raised in the peripheral blood of individuals with latent tuberculosis that does not progress (347, 348).

Similarly there was no trace of the association between TLR2 and IL-4 in cells from tuberculosis patients. It is worth noting that IL-4 downregulates TLR2 and also impairs signaling via TLR2 (43). Thus it is possible that the relationship we have found is characteristic of inactive memory cells, and is lost when these are activated. The minimal hypothesis suggested by our data is that memory Th2 cells that express IL-4 mRNA also express high TLR2 and low TLR1. This will be an interesting hypothesis for future study.

3.2. Signalling pathway of TLR and Th1/Th2 response

Signalling through TLR4 can also be associated with either Th2 or Th1 responses, and this appears to depend on the dose of endotoxin (TLR4 ligand) used (38). This dose effect might reflect the fact that TLR4 can drive activation of NFκB via a pathway involving the adaptor protein MyD88, or it can act via an alternative MyD88-independent pathway that involves interferon regulatory factor 3 (IRF-3) (21). Thus endotoxin causes MyD88-deficient DCs to support Th2 responses (349) .

In the case of TLR2 it is not clear that there is an alternative to the MyD88-associated pathway. However the diversity of effects of TLR2 might be related to the fact that it forms heterodimers with TLR1 or TLR6 (24, 224). The relative availability of these and perhaps of other potential dimerisation partners can affect function. For instance TLR2 transduces the response to phenol-soluble modulin, a factor secreted by *Staphylococcus epidermidis*. The response to this factor was enhanced by TLR6 but inhibited by TLR1, indicating a functional interaction between these receptors (350). The presence of hsTLR1 further complicates this issue (Chapter 5).

3.3. Future experiments

- These results are based on a single observation derived from analyzing a small number of donors (n=10) with no further functional assays. In order to validate this pilot result, a future study should recruit more subjects to validate the finding. Ideally, findings at the mRNA level should be by analysis of protein expression which could be done by flow cytometry. However potential problems exist in measuring membrane levels of TLR due to relative low

surface expression, donor variation and feedback control mechanisms (Discussed in Chapter 4, section 3.6).

- An alternative might be to use cell sorting to purify CD4 cells from normal healthy donors, expressing high TLR2 and low TLR1. These cells could stimulated with PMA/calcium ionophore, and compared with $TLR2^{low}$ $TLR1^{high}$ T cells from the same blood for expression of IL-4 of IFN-γ.

- The effect of IL-4 on the expression of TLR could be carried further *in vitro* by treating cells with recombinant IL-4 antibody, then challenge with M.tb antigen. The expression and activation of TLR can be assessed *in vitro*.

4. CONCLUSION

In conclusion, these results indicate an association between IL-4 and TLR2 expression and an association with the ratio of TLR1 to TLR2 in cells from healthy donors, but not from patients. The explanation is unknown, and the observation is based on a single experiment and technique. Nonetheless, present study provides a glimpse of the complexity of regulation mechanism of TLR2, and might indicate that resting memory Th2 cells that express IL-4 also express high TLR2.

Chapter 7. Pilot study of live *M.tuberculosis* infection in human PBMCs

1. INTRODUCTION

M. tuberculosis strains vary widely in the extent to which they spread in the population. This variation has mainly been attributed to differences in virulence of organisms, environmental conditions that affect the likelihood of inhaling aerosols as well as host genetics (351). Genes that are involved in initiation of protective cellular responses have long been acknowledged as essential in controlling Mtb replication within the host. For example, polymorphisms in Th1 cytokine surface receptors, pattern recognition receptors and Nramp 1 (natural resistance associated macrophage protein) were associated with susceptibility in infectious disease (352). Nramp 1 is expressed in the endosomal/lysosomal compartment of mouse macrophages and functions as a membrane cation pump (353). An important breakthrough in understanding host genetic factors and resistance to *M. tuberculosis* was recently reported. Pan and colleagues identified a candidate gene, Intracellular pathogen resistance 1 (Ipr1), which controls host resistance to tuberculosis in mice by reducing bacterial replication and programming cell death by apoptosis in infected mouse macrophages (76).

The virulence of a microorganism is defined as "the relative capacity of a pathogen to overcome host defence mechanisms" (354). Altering antigen processing or inhibiting APC maturation by lipoproteins of M.tb allows *M.tuberculosis* to subvert the protective cellular immune responses of the host (Chapter 2, section 2.9.4). Restriction

fragment length polymorphism (RFLP) analysis of clinical isolates reveals that a high percentage of the patients in developing countries result from recent transmission (60, 83) and strains from clusters were associated with rapid growth in human macrophages, suggesting that the capacity for replication may be a marker of virulence (351). The ability to replicate rapidly within macrophages was associated with early production of IL-10 and suppression of TNF-α in THP1 cells during the early stages of infection (355). Different *M. tuberculosis* strains were shown to exhibit differential pathogenesis by their ability to alter the host immunity through modified lipids (356). *In vitro* infection of monocytes with *M. tuberculosis* HN878 and related W/Beijing isolates preferentially induced interleukin-4 (IL-4) and IL-13, whilst the CDC1551 strain induced strong Th1 protective immunity (e.g. IL-12, TNF-α) (354, 356). Taken together, data suggest strain-specific virulence of *M. tuberculosis* may result from modulation of Th1/Th2 cytokine production.

The present study sought to determine expression profiles of TLRs and host immune response triggered by different organisms. The aims of this preliminary study were:

- To examine the effect of specific strains on expression of TLR4 and TLR2.

- To examine the involvement of TLR1, hsTLR1 and TLR6 in live organism infection.

- To assess the profile of Th1 (IFN-γ) and Th2 (IL-4) cytokines induced by the laboratory standard strain H37RV and Mexican clinical isolate strain CPA-96.

2. RESULTS

Mycobacterium tuberculosis H37Rv (ATCC 25618), the clinical isolate CPA-96 and the fast growing environmental saprophyte *M. vaccae* (NCTC 11659) were grown in Middlebrook 7H10 agar (Difco). Mycobacteria were disaggregated by vigorous vortexing with glass beads. After brief centrifugation to deplete aggregates of organisms, organisms were counted in improved Neubauer haemocytometers. PBMCs from 5 healthy control donors were prepared and infected with living mycobacteria at a dose of approximately 1 organism per macrophage (~10% monocytes in PBMC). 2-way ANOVA with Bonferroni posttests and correction were employed for the analysis.

7.1. HuPO stability in PBMC infected with live mycobacteria

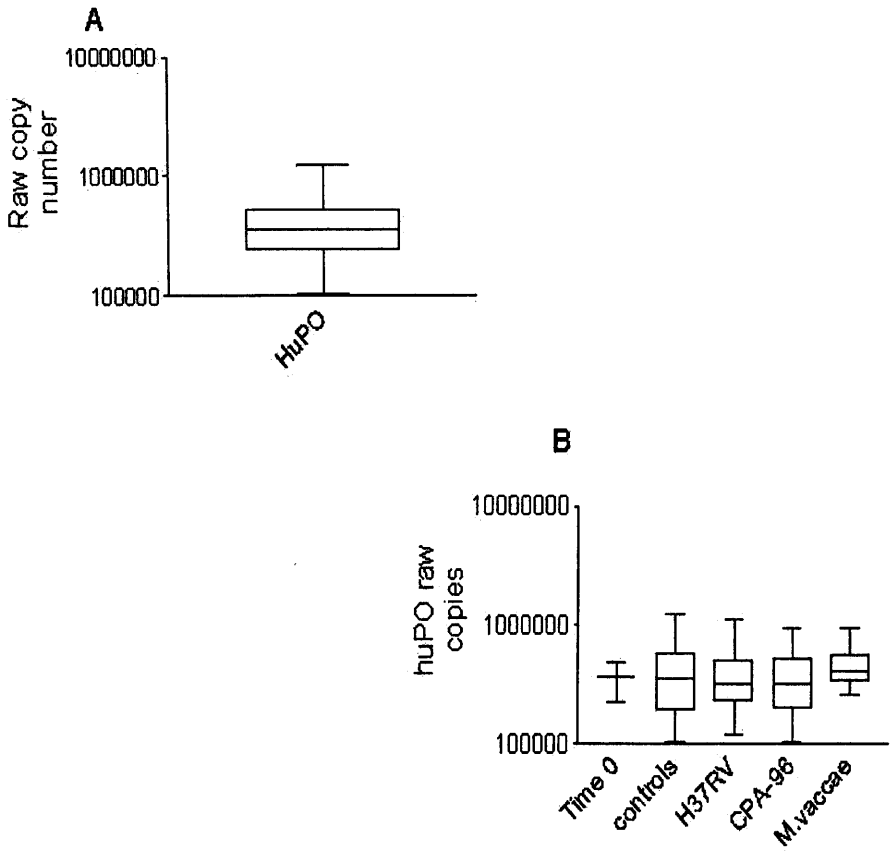


Figure 7.1. Stability of huPO in cells infected with living mycobacteria *in vitro.* PBMCs from normal donors (n=5 except M. vaccae which is n=3) incubated with live mycobacteria, Mtb H37Rv, Mtb CPA-96 and M. *vaccae*, at a dose of approximately 1 organism per macrophage and cells were harvested at 18 hrs, 24hrs, 48 hrs and 66 hrs post-treatment. (A) Shows overall stability of huPO under the experimental condition (coefficients of variation = 62.3%). (B) shows the effect of antigens on the stability of huPO over the treatment period. Incubation cells with living M. *vaccae* caused least variation among the treatments (coefficients of variation = 41.5%) compared to controls (71.9%), H37Rv (72.1%) and CPA-96 (60.1%). Results are conducted in triplicates and are presented as median ± 25th and 75th percentiles.

7.2. Effect of different strains on expression of TLR2 and TLR4 in human PBMCs.

We investigated whether the effects seen in the *ex vivo* studies with patients' cells could be reproduced by infecting PBMC from normal donors with living mycobacteria at a dose of approximately 1 organism per macrophage. Expression of TLR2 increased in PBMC after 48 hours in culture even in the absence of mycobacteria. However there was a significant further increase in TLR2 mRNA in the presence of H37Rv at 48hrs ($p < 0.01$) and 66hrs ($p < 0.01$) (Figure 7.2. A). H37Rv did not cause changes in expression of TLR4 (B) and TLR7 (C). The environmental saprophyte, *M. vaccae,* caused a distinct pattern of changes with inhibition of TLR4 at 48hrs ($p < 0.001$) and 66hrs ($p < 0.01$) (B). Notably, the clinical isolate strain CPA96 had no effect on the expression of TLR2 or TLR4 (B & C)

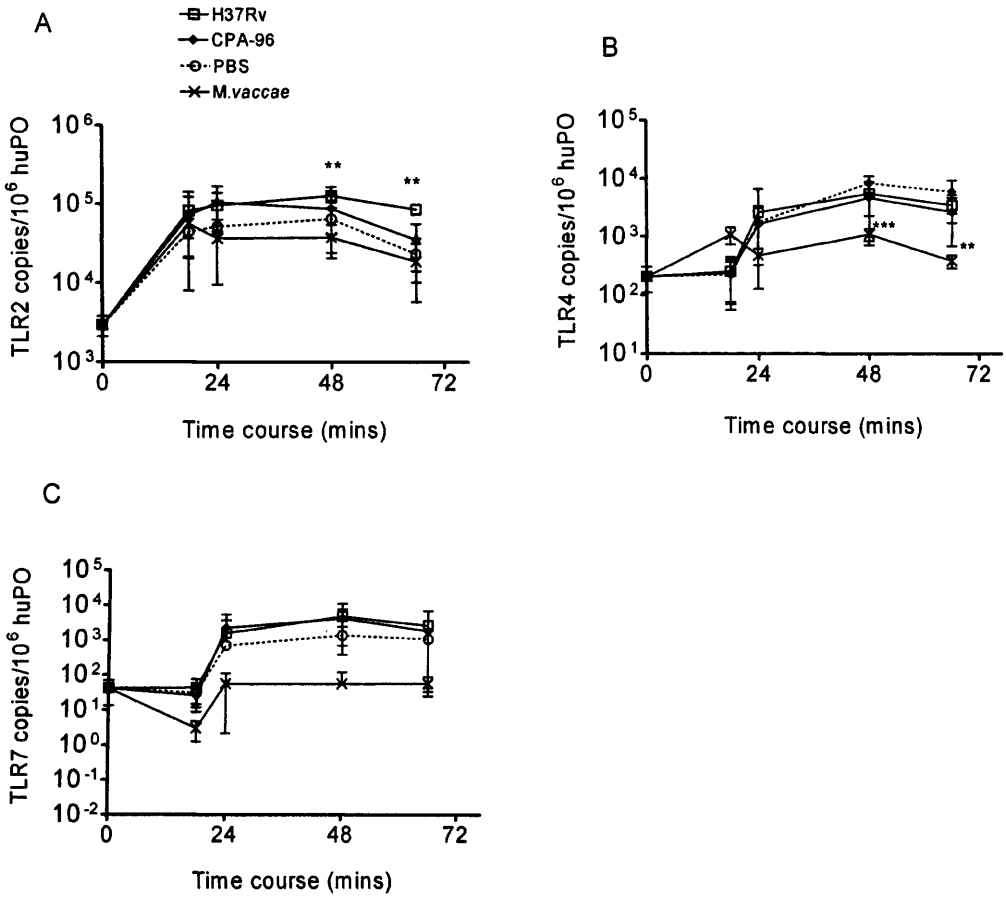


Figure 7.2. Kinetics of expression of TLR2 and TLR4 in human PBMCs in response to incubation with living Mtb H37Rv, Mtb CPA-96 or *M. vaccae*. Incubation with H37Rv increased TLR2 gene expression at 48hrs and 66 hrs (both $p < 0.01$) compared to control wells (A), but incubation with *M. vaccae* and clinical isolate CPA-96 did not. By contrast H37Rv did not cause any change in expression of TLR4, whereas *M. vaccae* tended to suppress TLR4 at later time points at 48hrs ($p < 0.001$) and 66hrs ($p < 0.01$) (B). Results are from healthy control donors (n=5 except *M.vaccae* which is n=3) and are presented as means ± SD.

7.3. Effect of different strains on expression of TLR2 dimerisation partners

Expression levels of co-receptors of TLR2; TLR1 and TLR6 were also not changed (C, E), but there was an increase in expression of hsTLR1 after 66 hours culture with H37Rv (A, $p<0.05$). A trend towards suppression of TLR1 (B) (not significant) and an early increase in expression of hsTLR1 (A) ($p<0.05$) were also observed in wells incubated with *M. vaccae*.

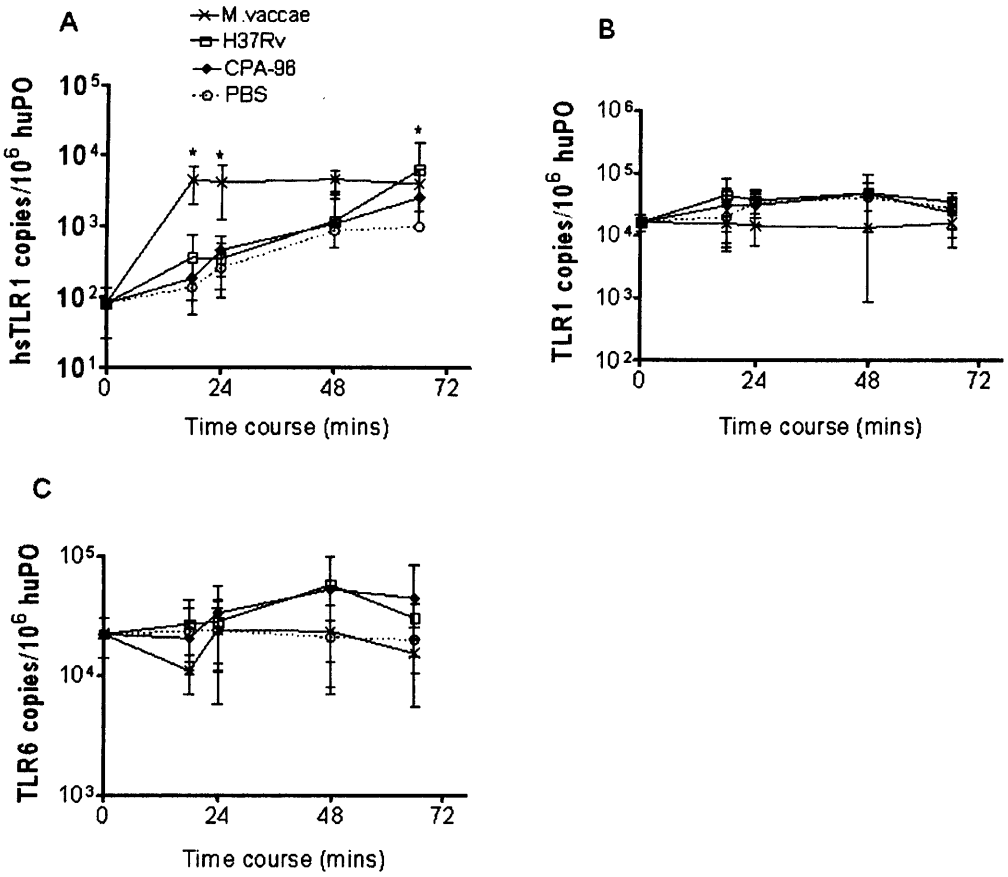


Figure 7.3. Kinetics of expression of potential TLR2 heterodimerisation partners (TLR1, hsTLR1 and TLR6) in human PBMCs in response to incubation with living Mtb H37Rv, Mtb CPA-96 or *M. vaccae*. M.tb increased expression of the gene encoding hsTLR1 at 66hrs postreatment ($p<0.05$) whilst *M. vaccae* induced increased hsTLR1 gene expression at 18 hrs ($p<0.05$) and

24 hrs post-treatment ($p<0.05$) compared to control wells (A). By contrast, *M. vaccae* suppressed the rise in expression of TLR1 (B) that occurred over time in control wells or wells containing H37Rv or CPA-96. Results are from healthy control donors (n=5 except M. vaccae which is n=3) and are presented as means ± SD.

7.4 Th1 (IFN-γ) and Th2 (IL-4) cytokine profile: qPCR and ELISA

The CPA-96 strain was a gift from Dr. Hernandez-Pando. It is a very virulent strain responsible for an outbreak in Mexico, but it is not a Beijing strain. Nevertheless, like Beijing strains a preliminary study in mice showed that it preferentially induced IL-4 and impaired IFN-γ production at the late stage of infection (Dr. Hernandez-Pando's unpublished data). The second objective of this study was therefore to assess the Th1/Th2 cytokine profile in human PBMCs induced by Mtb H37RV and CPA-96.

The gene encoding IFN-γ was rapidly induced in wells stimulated with H37Rv at 18hrs post-treatment ($p<0.01$) following a second peak at 48 and 66hrs post-treatment (all $p<0.01$) compared to controls (Figure 7.4.A). In contrast, treatment with CPA-96 resulted in a delayed response at 48 hrs ($p<0.05$) and 66 hrs post-treatment ($p<0.01$) whilst *M. vaccae* only showed a difference at 48hrs post-treatment ($p<0.01$) (Figure 7.4.A). The early induced IFN-γ expression by H37Rv was correlated with supernatant protein level at 18 hrs ($p<0.05$), 24 hrs (($p<0.05$) and 66 hrs ($p<0.01$) measured by ELISA (Figure 7.4.B). Protein level induced by CPA-96 only showed differences at 66 hrs post-treatment ($p<0.01$) by ELISA (Figure 7.4.B). Although treatment of M. *vaccae* resulted in increasing mRNA encoding IFN-γ at 48 hrs (Figure 7.4 A), this was not shown in the protein level (Figure 7.4.B).

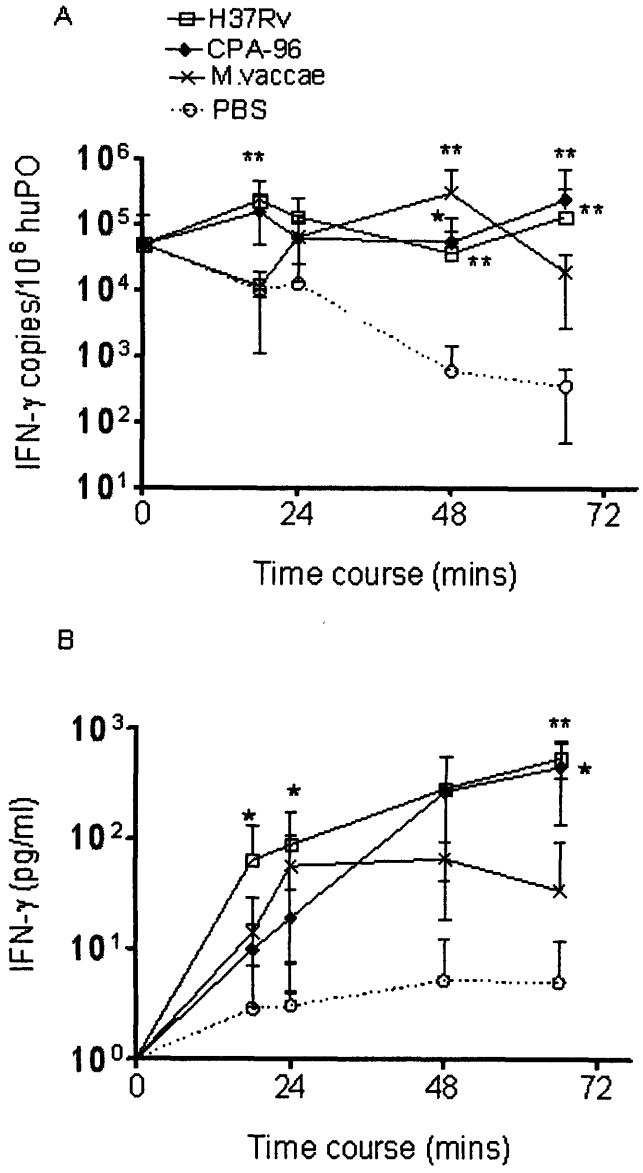


Figure 7.4. IFN-γ levels in human PBMCs treated with living Mtb H37Rv, Mtb CPA-96 or *M. vaccae*. Wells treated with H37Rv induced an early IFN-γ response at 18hrs ($p<0.01$), 48hrs ($p<0.05$) and 66hrs ($p<0.01$) whilst wells incubated with CPA-96 showed a delayed response at 48 hrs and 66hrs post-treatment ($p<0.05$ & $p<0.01$ respectively) (A). The early IFN-γ expression induced by H37Rv correlated with the supernatant protein level; in contrast, wells incubated with CPA-96 showed delayed protein secretion at 66hrs post-treatment ($p<0.05$) measured by ELISA (B). Despite increased IFN-γ mRNA expression at 48hrs ($p<0.01$) in wells treated with *M.*

vaccae (A), the mRNA level did not correspond to an increased protein level (B). Results are from healthy control donors (n=5 except *M. vaccae* which is n=3) and are presented as means ± SD.

Since differential pathogenesis of CPA-96 was linked to the increasing mRNA level of IL-4 in infected mice, the genes encoding IL-4 and IL-4δ2, which is an antagonist of IL-4, were assessed. Figure 7.5 shows that no differences were observed in mRNA encoding IL-4 (A) or IL-4δ2 (B) over the investigated periods in wells incubated with H37RV, CPA 96 or *M. vaccae* when compared to control wells.

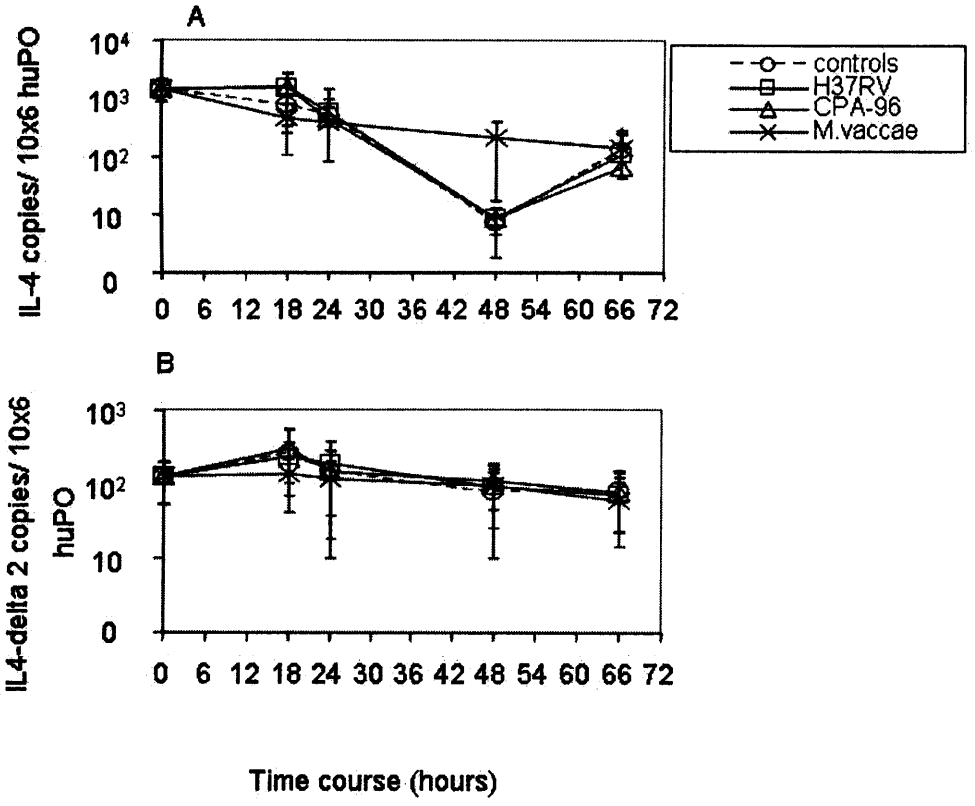


Figure 7.5 Kinetics of expression of IL-4 (A) and IL-4δ2 (B) in human PBMCs stimulated with living Mtb H37Rv, Mtb CPA-96 or *M. vaccae*. Results are from healthy control donors (n=5 except *M.vaccae* which is n=3) and are presented as means ± SD.

3. DISCUSSION

It is emphasised that this was a pilot experiment performed only once. Thus all discussion must be tentative.

3.1. Effect of viable M.tb bacilli on expression of TLR2 and TLR4

There are currently no data that we are aware of examining the effect of live M.tb bacilli on the expression of TLR in human cells. One published paper reported that viable H37Rv confer responsiveness via TLR2 and TLR4 in the CHO fibroblast cell line that were stably transfected with plasmids encoding human CD14 and human TLR2 or TLR4 (297). The present study showed a late response of TLR2 to H37Rv but not to CPA-96, suggesting a strain-specific immune response (Figure 7.2 A). The delayed increase in TLR expression may indicate differences in the nature of the antigen. Sonicated H37Rv induced a rapid expression of TLR2 at 18hrs post-stimulation in THP1 cells (Chapter 4, section 2.7) whilst live H37Rv induced TLR2 after 48hrs post-treatment (Figure 7.2 A). Neither sonicated H37Rv nor viable H37Rv/CPA-96 induced expression of TLR4 *in vitro* in THP1 or in human PBMCs respectively. This is in contrast to the clinical result in which up-regulation mRNA encoding for TLR4 was observed in unstimulated whole blood in PTB patients (Chapter 4, section 2.3). This might indicate that expression of TLR4 at the mRNA level is sensitive to increased circulating levels of proinflammatory cytokines, but not to mycobacterial components. The role of TLR4 in combating *M. tuberculosis* infection remains controversial in genetic defect mice model (Chapter 1, section 2.9.2).

3.2. strain-specific cellular immune response

Certain *M. tuberculosis* strains (e.g. W/Beijing isolates) were reported to down-regulate expression of proinflammatory cytokines/chemokines and to favour the development of Th2 cytokines, such as IL-4 (356). H37Rv induced rapid IFN-γ protein release as early as 18hrs post-treatment. In contrast CPA-96 induced delayed response only at 66hrs post-treatment (Figure 7.4), confirmed both at the mRNA and protein levels. Although preliminary results in mice infected with CPA-96 strain showed preferential expression of IL-4 and impaired IFN-γ (Dr. Hernandez-Pando's unpublished data), the present study did not detect any differences in the mRNA encoding IL-4 or IL-4δ2 (Figure 7.5).

3.3. *M. vaccae* and cellular immune response

The effect of living *M. vaccae* on the expression of TLR and cytokine expression was greatly obscured by the small sample size (n=3) plus great donor variation. Nonetheless, *M. vaccae* seems exert a post-translational regulation where it allows *M. vaccae* to switch off IFN-γ protein production despite production of the mRNA.

M. vaccae is a harmless environmental mycobacterium which has shown particular therapeutic potential. Treatment with *M. vaccae* significantly reduced murine allergic pulmonary inflammation by inducing $CD4^{+}CD45RB^{low}$ regulatory T cells that secret IL-10 and TGF-β (357). In a subsequent study, it was reported that *M. vaccae*-treated allergic BALB/c mice developed high levels of expression of IL-10, TGF-β and IFN-α in pulmonary CD11c+ cells, likely to be DCs (119). Instead of increasing the Th1 cytokine level, treatment with *M. vaccae* induced a regulatory immune response that switched off the local Th2-mediated inflammation. *M. vaccae* is also part of a TB

treatment regime, together with antibiotics, in China where it is claimed that it shows efficacy in treatment of multiple-drug resistant TB (358, 359). Treatment regimes combined with *M. vaccae* increased the ratio of $CD4^+/CD8^+$ T cells, sputum negative conversion and improved X-ray manifestations in patients with pulmonary tuberculosis, thought there is currently no information on the relapse rate after successful treatment (358, 360).

3.4. Future experiments

Despite the inevitably small sample size, this pilot study showed some interesting preliminary results of cellular immune responses to different mycobacterial strains. Furthermore, the modulatory response triggered by the environmental saprophytic *M. vaccae* deserves further investigation.

- Future experiment should increase sample size and extend incubation time points in order to clarify whether viable M.tb is able to induce expression of TLR2 and TLR2 co-receptors: hsTLR1, TLR1 and TLR6 at later stages of infection.

- Other cytokines that have been implicated in directing immunosuppressive immune response such as IL-10, TGF-β and IFN-α should also be included in the future study, particularly in view of the strikingly different response to *M. vaccae* which is know to induce regulatory T cells. Although the role of regulatory T cells in patients with pulmonary tuberculosis in unknown,

accumulating evidence from other chronic diseases suggests an important role for regulatory cytokines (e.g. IL-10, TGF-β and IFN-α) (43, 119).

- Characterise which components of CPA-96 possess immunosuppressant property as well as the underlying mechanisms.

4. CONCLUSION

In conclusion, both sonicated H37Rv (Chapter 4, section 2.7) and viable H3R7v are able to induce expression of TLR2 mRNA, suggesting that TLR2 plays a central role in the host defence against *M. tuberculosis* infection. It remains unclear which components of M.tb initiate the upregulation of TLR2. Strain-specific immune response was also observed in the present study. Infection of PBMCs with the clinical isolate CPA-96 did not trigger increased expression of the gene encoding TLR2 at the end of incubation. This delay was accompanied by delayed production of IFN-γ. Future experiment should address the question whether the CPA-96 strain uses TLR2 to subvert the innate immune response to escape host killing.

Chapter 8. General Discussion

1. INTRODUCTION

1.1. Summary of results

Identification of biomarkers for diagnosis and assessment of disease progression in patients with tuberculosis is urgently needed. The potential of TLR was explored in the present study because they play an essential role in the interaction between the host and *M. tuberculosis.* Changes in expression of TLR might reflect changes in immunological profile during disease progression. Since this project mainly studied gene expression, factors (e.g. reference gene stability, qPCR efficiency, RNA quality and normalisation methods) that can contribute to biased results were carefully optimised prior to undertaking the investigation. This is of particular importance for genes that are expressed at, and are biologically active at very low levels, such as TLR and IL-4.

Overall, the results of this project suggest that up-regulation of mRNAs encoding TLR2, TLR4 and TLR2 heterodimerisation partners (hsTLR1, TLR1 and TLR6) in peripheral blood might represent useful markers in the study of patients with progressive TB and their contacts. TLR2 and its heterodimerisation partners showed the most responsiveness to *M. tuberculosis* antigens, though it is not known which components of M.tb caused these effects, or which receptors they signalled through to

cause upregulation of TLR (Clearly the TLR themselves are a possibility, that is partially supported by the results seen with Pam3CysK4).

Expression of TLR is controlled at multiple stages. Distinct expression profiles of TLR in two compartments of immune cells (whole blood v.s. BAL cells) in patients with progressive tuberculosis suggest that regulatory mechanisms exist at the site of infection. This is likely to be influenced by local inflammatory cytokines (Th1, Th2) and by regulatory cytokines that counterbalance the tissue damage triggered by TLR. Secondly, *M. tuberculosis* controls expression of TLR at a post-transcriptional level, via a mechanism that controls mRNA ½ life which is p38 MAPK-independent. Finally, M.tb induced an increase in alternative splicing of TLR1. This may result from the different usage of transcriptional promoters, and could theoretically lead to changes in the activation of TLR2.

1.2. Mechanisms controlling alternative splicing of TLR

Initiation of transcription is controlled by regulatory elements such as promoters and transcription factors (317). There are three types of promoter sequences identified in eukaryotes and each exerts differential regulatory function on initiation of transcription (Details see Figure 8.1). These sequences are predominantely located in the 5'-UTR region, ranging from 20 bp to 50 kb upstream of transcription start sites. Differential promoter usage among members of TLR accounted for the cell-type specificity (361), species variation among men and mice (247) and the diversity of alternative transcripts (266).

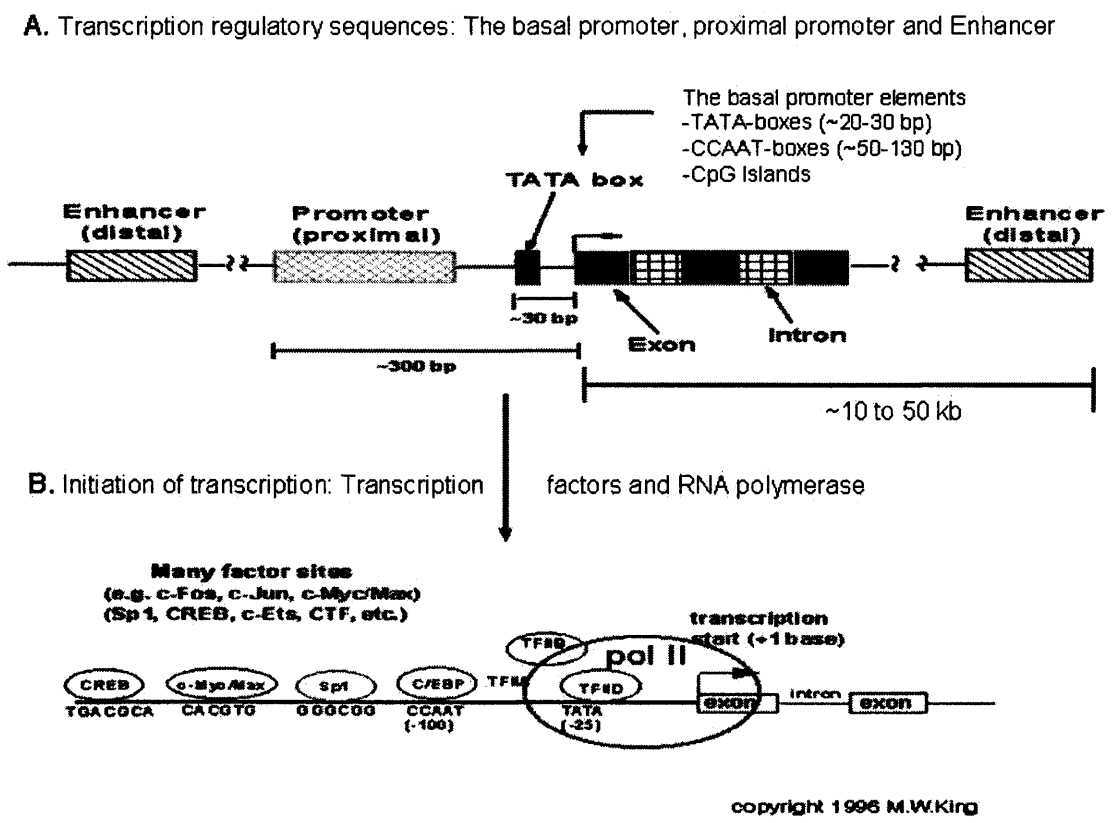


Figure 8.1. Diagram of Initiation of transcription in eukaryote.

A. shows three types of promoter sequence identified in eukaryotic DNA: The basal promoter elements, proximal-promoter regions and Enhancer/Silencer.

- The basal promoter elements (TATA boxes, CCAAT boxes and CpG islands) determine the site of transcription initiation and direct binding of RNA polymerase II.

- Promoter-proximal elements (~20bp to 300bp) may help regulate a particular gene. Typical features of a myeloid-specific gene lack a TATA-box and contain several purine-rich sequences that are recognised by the myeloid and B cell-specific transcription factor PU.1. This is the case for human TLR2 and TLR4 (247).

- Enhancers (~8 to 20 bp) position RNA polymerase II to initiate transcription at the start site and influence the rate of transcription. Enhancers may be either upstream or

downstream and as far away as 10 to 50 kb from the transcription start site. Proximal-promoter elements and enhancers often are cell-type specific (362).

B. Regulatory sequences interact with specific transcription factors to initiate transcription. Various types of RNA polymerase (I, II, III) and transcription factors are used depending on types of RNA being transcribed. (Figure 8.1 is modified from M.W. King (http://web.indstate.edu/thcme/mwking/gene-regulation.html#initiation)).

Results of half-life experiments in this study suggest that the observed change in the ratio of hsTLR1 to TLR1 triggered by Mtb antigen was not due to alteration of the hsTLR1 half-life (Chapter 5, Figure. 5.4). This indicates that alteration in the ratio must be controlled at the level of splicing or transcription, depending on whether the transcribed preliminary mRNA of hsTLR1 is the same as that of TLR1 or not. Since the present study did not obtain the complete $5'$ sequence, we can not conclude whether the observed alternative splicing is due to variations in 5' promoter usage. However, most alternative splicing decisions involve competition among potential splice sites (317). Therefore, splicing patterns of TLR induced by *M. tuberculosis* can be controlled by any mechanism that alters the relative rates of splice site recognition as well as by the use of multiple transcription start sites. The recognition of splice sites is governed by splicesomes which recognise GT-AG acceptor/donor sites. Several enhancer/suppressor elements can regulate this process such as consensus sequence, cis-acting elements and trans-acting factors (317). More experiments are required to address this question (Discussed in detail in Chapter 5, section 3.6.2).

1.3. Alternative activation of macrophages

Bronchoalveolar macrophages constitute more than 80% of lavage cells and their activation status may reflect the immunopathology in the lung. Exposure of macrophages to either Th1 (IFN-γ) or Th2 cytokines (IL-4 and IL-13) induces two distinct pathways of activation (363). The term 'Alternative activation of macrophages' was defined by Gordon S to describe the fact that IL-4 and IL-13, but not IL-10, promote a distinct phenotype of macrophage activation that differs from classical IFN-γ-dependent macrophage activation (98). Although IL-10 was shown to promote the alternative activated phenotype *in vitro* (364), it is unclear whether IL-10 promotes alternative activation or suppresses macrophage function (e.g. down-regulated co-stimulators) (98). Phenotypes of activated macrophages differ in their effector function, surface receptor, chemokine and cytokine expression (summarised in Table. 8.1) (365 646).

As discussed in Chapter 1 (section 2.12 & section 2.3), classical IFN-γ-dependent macrophage activation is essential in controlling intracellular pathogens in the early phase of infection by orchestrating antimicrobial mechanisms and containing pathogens within granulomas. However, this control mechanism may fail to contain the pathogens and excessive inflammation may lead to pathological consequences in local host tissue (Discussed in section 2). Alternative activation of macrophage has been proposed as a mechanism by which Type 2 responses attenuate excessive inflammation (98). It has been demonstrated in vitro that IL-4 down-regulates surface TLR2 expression and alters TLR2-mediated signalling (43). Questions that remain to be further investigated include whether the expression and activation of TLRs were modified by the presence of IL-4 in the BAL in patients (6). Although T_H2 responses

are clearly responsible for alternative macrophage activation, the importance of this activation pathway in T cell priming, differentiation, or effector function is largely unknown.

Table 8.1. Phenotypes of macrophages induced by Th1 or Th2 cytokines (365 646).

Markers	Classical activated macrophages	Alternative activated macrophages
Induced agents	IFN-γ and LPS	Il-4, IL-13
Surface receptors	-TLR2 and TLR4 - CD80 and CD86 -Fcγ-RI, II, III (CD16,32,64; opsonic receptors by coating a particle with proteins to facilitate phagocytosis)	-CD23 (Fcε-RII for IgE) - Mannose receptor (non opsonic receptor) -CD163 (The hemoglobin-haptoglobin scavenger receptor & function for the resolution inflammation)
Cytokines	-TNF-α, IL-6, IL-1, IL-12 and IFN-γ	- IL-10, IL-1 receptor agonist
Cytokine receptor	IL-1 type I receptor	IL-1 type II decoy receptor
Chemokines	-CXCL 8, 9, 10, 11 -CCL 2, 3, 4, 5	-CCL 17, 18, 22, 24
Chemokine receptors	-CCR 7	-CXCR 1 & CXCR 2
Metabolic factors	-iNOS (inducible nitric oxide synthase) -ROI	-L-arginase 1 (counteracts NOS2) -12,15-lipoxygenase

1.4. Current diagnostic methods for latent tuberculosis

T cell mediated IFN-γ is a crucial mediator for protection against tuberculosis. The existing diagnostic assays for latent TB infection are all based on the capacity of memory T cells to initiate rapid cell-mediated immunity when they encounter specific antigens (e.g. tuberculin skin test {TST}, ELISPOT or IFN-γ ELISA assay). The major differences between the classical TST method and newly developed assays are the specificity due to the different antigens used {purified protein derivative (PPD) v.s. early secreted antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10)}. PPD shows cross-reactivity with BCG and environmental bacteria whilst ESAT6/CFP-10 are relatively *M. tuberculosis* specific. Both ESAT-6 and CFP-10 are encoded on the region of difference domain 1 (RD1), a region associated with virulence and attenuation of BCG (366). So the cross-reactivity with BCG and environmental microbacteria are excluded but they may react to components of close species such as *M. marinum, M. szulgai* and *M. kansasii* and *M. leprae* (367). One problem of IFN-γ based assays is anergy, which frequently occurs during active tuberculosis. IFN-γ measurements do not reflect the degree of protection against tuberculosis and cannot be used as markers for disease progression.

2. ACTIVATION OF TLRs AND IMMUNOPATHOLOGY

Pathogen-induced immune responses have long been acknowledged as double-edge swords. A effective immune response is required for the elimination or control of the invading organisms but an excessive immune response can also cause host damage. Neutralisation of TLR by mouse antibody abolished proinflammatory cytokine production (e.g. TNF-α, IL-12) in cells treated with mycobacterial components (24, 218, 227). Similarly, $MYD88^{-/-}$ DCs failed to activate CD4+ T cells *in vivo* (296). Those

studies have firmly established the important role of TLR in eliciting innate and adaptive immunity. On the other hand, this also suggests that activation of TLR is responsible for the undesirable effects of infection-related tissue damage. These include the development of fever, shock, necrosis, fibrosis and tissue injury. Furthermore, accumulating evidence suggests that nucleic acids derived from pathogens or damaged host tissue can trigger the activation of TLRs which may lead to subsequent autoimmunity (368). In order to avoid host damage, mechanisms used by innate immune cells (e.g TLRs and other PRRs) to initiate such a strong protective immunity are tightly regulated (21). This is best characterized in the activation of TLR4 and TLR2 and will be discussed below.

2.1. Regulation of TLR4 induced by LPS

LPS is known to cause this dual effect in the host by excessive production of proinflammatory cytokines, such as TNF-α and IL-12. An excessive response to LPS is known to induce septic shock in the host. In contrast, subsequent challenge to LPS after an initial exposure, sometimes induces a suppressive immune response called 'endotoxin tolerance'. This paradoxical immune response induced by LPS is reflected in contradictory data on the effects of LPS on expression of the TLR4 gene. Delivery of LPS to TLR4 requires the accessory proteins LBP (LPS-binding protein; found in serum), CD14 and MD-2 (the latter two proteins can exist in soluble form, or bound to the membrane or TLR itself). Muzio and colleagues reported that LPS increased expression of mRNA encoding TLR4 in human peripheral blood monocytes in an ActD-dependent manner, suggesting transcriptional regulation (200). However, Poltorak and Nomura separately reported that treating murine macrophages with LPS resulted in reduced TLR4 expression at both mRNA and protein levels (189, 369). Fan

and colleagues reported that expression of TLR4 was destabilized after challenge with LPS in a murine model, and both transcription activity and RNA stability contributed to the expression level of TLR4 in mouse macrophages (306). Specifically, while LPS was shown to increase transcription activity of TLR4, destabilization of TLR4 transcripts was switched on in parallel, thereby reducing the net effect on TLR4 transcripts following cellular activation. Excessive LPS responses can also be down-regulated by a naturally occurring soluble form of mouse TLR4, designated smTLR4 (268). Therefore, evidence from murine studies may explain rapid down-regulation of the gene encoding TLR4 in murine cells. It remains unclear whether those mechanisms apply in humans.

2.2. Regulation of TLR2

TLR2 is the main receptor for ligands of *M. tuberculosis* because it binds lipoprotein, lipopeptide, PGN, LTA, and LAM all of which are components of mycobacteria (22). Heterodimerisation of TLR2 to TLR1, TLR6 and TLR10 allows TLR2 to recognise such a broad spectrum of ligands (Chapter 1, section 3.4.3), though the ligand of TLR10 remains unidentified. Accordingly, the activation status of TLR2 was found to be influenced by it's partners (Chapter 1, section 3.4.4). A soluble form of TLR2 (sTLR2) can also act as a feedback mechanism to inhibit membrane bound TLR2 induced by a Lipoprotein of Mtb (269). The regulatory mechanisms that control mouse and human TLR2 isoforms differ in that smTLR4 was regulated at the posttranscriptional level, whereas sTLR2 was regulated at a posttranslational level. Although the specific interfering mechanism is unclear, it is likely that soluble TLRs interfere with the ligand binding capacity of membrane-bound TLRs either by interacting with CD14 or by competing for ligand.

2.3. Feedback mechanisms in the signaling pathway

Other negative regulation mechanisms seem to exist in the downstream signaling pathway. Several molecules such as MyD88s and IRAK-M that are involved in the TIR signaling pathway have been reported to play a role in feedback regulation in LPS induced tolerance (370, 371). An alternatively spliced form of MyD 88 (MyD88s) is induced by LPS treatment of monocytes, and by excising it's intermediate domain (ID domain) it prevents the association of IRAK-4 to MyD88 resulting in the inactivation of NF-kB (370).

3. CLINICAL IMPLICATION

3.1.TLRs and asthma/allergy

TLR agonists are by their nature pro-inflammatory and those that have Th1 adjuvant activity have been considered as candidates to turn off excessive humoral IgE and Th2 cytokines in asthma/allergy patients. Polymorphisms of TLR2 and TLR4 were originally reported to be associated with susceptibility to septic shock (28, 31). Since then, numerous diseases have been linked with polymorphisms in all the members of the TLR family, ranging from prostate cancer (372), type I diabetes (373), leprosy (27, 250), inflammatory bowel disease (374) and allergy (248, 249, 375). A polymorphism in TLR10 associated with asthma was recently reported (376). The protective effect of TLR2 and TLR4 in the risks of developing asthma/allergy has been one of the focuses. Early childhood exposure to endotoxin showed an inverse correlation with asthma/allergy in European children (377) and led to the identification of the key genes (CD14, TLR2, TLR4) responsible for this association (248, 249, 375, 378). However, published papers based on associations between diseases and single nucleotide

polymorphisms (SNP) provide inconclusive results, which is also the case for asthma and TLRs (379-381). These coincide with recent reports which suggest that ligands of TLR (LPS and Pam3cysK4) used as Th1 adjuvant induces Th2 response (Discuss in Chapter 6) (37-40, 42).

3.1.1. TLR9 as potential immunotherapy for asthma/allergy

In fact, synthetic CpG-oligodeoxynucleotides (CpG-ODNs) showed the most promising potential as immune adjuvants for allergy treatment and cancer therapy. TLR9 recognises unmethylated CpG motifs present in bacterial and virus DNA and drives a potent Th1 response, with strong establishment of Th1-type memory T-cells (382). Plasmacytoid DC (pDC) selectively expresses TLR9 and is stronger producers of IFN-α, which is important for anti-viral effects as well as for induction of regulatory T cells. Activation of pDCs by TLR9 agonists induces regulatory T-cells (383). TLR9 also activates DC-independent pathways resulting in the local production of IDO (indoleamine 2,3-dioxygenase) in the lung and suppressing T-cell activity (384). Thus administration of allergen complexed with TLR9 agonists may provide a potent mechanism of desensitizing established atopic responses and inhibit allergic responses (385). However, TLR9 is potentially associated with autoimmune diseases, because of the nature of its ligand. MyD88-dependent activation of TLR9 has been implicated in the activation of autoreactive B cells *in vitro* (386). Later, it was shown that the generation of anti-dsDNA and anti-chromatin autoantibodies is specifically inhibited in TLR9-deficient lupus-prone mice (387). ODN containing no CpG-motifs activated the innate immune system *in* a TLR9 dependent manner, suggesting that TLR9 can recognise self-DNA (388, 389). However, a genetic study did not find any association between polymorphisms of TLR9 and the risk of systemic lupus erythematosus (SLE)

(390, 391). A key issue in the future use of TLR9 as an immunotherapeutic agent for asthma/allergy will be the need to avoid autoimmunity when delivering TLR9 ligands to the host.

3.1.2. Immunotherapeutic effect of *M. vaccae* in asthma/allergy

Another vaccine that shows promising immunotherapeutic efficacy in patients with asthma is the saprophytic species *Mycobacterium vaccae*, which works via an immunoregulatory pathway, rather than by inducing a Th1-bias. Administration of *M. vaccae* to BALB/c mice before, or even after sensitisation with ovalbumin significantly reduced Ag-induced airway hyper-reactivity by increasing antigen-specific regulatory T cells (357, 386, 392) and regulatory cytokines (e.g. IL-10) (119). This is in phase II clinical trials. TLR2 has been found to be expressed in regulatory T cells (206) and TLR2 ligands can induce IL-10 secretion (393, 394). Preliminary studies in THP1 cells incubated with sonicated *M. vaccae* showed no change in expression of TLR2, despite increased expression of genes encoding regulatory cytokines (e.g. IL-10, IFN-α). TLR2 partners (hsTLR1, TLR1 and TLR6) were also expressed at increased levels in response to sM.v treatment. Most striking is the down-regulation of TLR4, which is even more profound in PBMCs cultured with live *M. vaccae* (Chapter 7). Pasare and Medzhitov demonstrated that microbial induction of the Toll pathway blocked the suppressive effect of CD4+CD25+ regulatory T cells, allowing activation of pathogen-specific adaptive immune responses. (395). This suppressive effect is also required IL-6. Therefore, suppression of TLR4 expression may facilitate or enhance the function of *M. vaccae*-specific regulatory T cells. (396) Ligands of TLR2 and TLR4 can both induce IL-6 secretion (393, 396). Possibly upregulation of the heterodimerisation partners of TLR2 induced by *M. vaccae* leads to the production of IL-6. This requires investigation.

3.2. Immunotherapy and vaccine design in tuberculosis

3.2.1. BCG vaccine

BCG is the most widely used vaccine worldwide but its efficacy in protection against tuberculosis is variable, ranging from zero to 80% in adults or adolescents (397). However BCG protects against severe forms of childhood TB, especially meningeal TB. Its protective efficacy progressively wears off during adolescence and the vaccine does not protect against pulmonary TB in adults (397). Currently, more than 120 TB vaccine candidates have been developed, most of which are selected by their Th1 inducing ability with different design strategies. The validity of the view that vaccines designed for maximal Th1-boosting capacity are the most likely to work has been challenged (17, 73, 397). Similarly, clinical evidence shows no sign of a defect in Th1 cytokine level in the lungs of patients, while there is a rising level of Th2 cytokines (IL-4) in patients with pulmonary tuberculosis (6). A paradox of immunopathology was proposed for the failure of BCG (16).

3.2.2. TLR2 and potential immunotherapy

Although the dominant response to mycobacteria is Th1-biased, an immune sabotage phenomenon by Mtb is observed (Chapter 1, section 3.6). TLR2 and DC-SIGN are the key molecules implicated in this paradox and the underlying regulatory mechanisms are also being studied (Chapter 1, section 3.6). In the case of TLR2, activation of TLR2 is likely to be controlled in some way by its heterodimerisation partners (Chapter 1, section 3.4.4). The fact that hsTLR1 showed the greatest response to M.tb in the present study complicates this matter further and requires further investigation. Until the underlying signalling pathway of TLR2 is understood, the use of ligands of

TLR2 (e.g. 19 kD lipoprotein, 24 kD lipoprotein, LAM) as adjuvant is limited. Nonetheless, progress in understanding in immune sabotage mechanisms used by *M. tuberculosis* will certainly help to design a better immunotherapy for tuberculosis. This is particularly important in tackling latent infection, which is characterised by reactivation of disease from the latent state. The molecular mechanisms are not known.

3.2.3. Approaches for new vaccines

Control of tuberculosis may need not only one but two or more vaccines. This is based on the characteristics of immunity to TB infection. Early immunisation is required to prevent the onset of the disease. A different vaccine might be required to prevent disease reactivation in latently infected individuals. Yet another type of vaccine might be active as an immunotherapeutic, to shorten therapy, or to treat multiple drug resistant TB. A further complication is the fact that a live vaccine might need to be able to proliferate enough to be immunogenic, despite a degree of previous immunity induced by environmental mycobacteria or BCG. Efficacy of a new vaccine should be tested in different geographic latitudes with different degrees of pre-exposure priming to non-pathogenic mycobacteria (17).

4. CONCLUSION

Understanding how host genetic factors interact with mycobacteria and the initiation of the subsequent immune response is important for designing effective vaccines and immunotherapies for tuberculosis. The optimum effector response requires signals from TLR, but TLR may be used as means for immune subversion by *M. tuberculosis*. The present study demonstrated that TLR expression differs in different immune compartments (peripheral blood v.s. Bal) suggeasting a control mechanism at the site of infection. *M. tuberculosis* exerts regulatory effects on TLR expression at transcriptional and posttranscriptional levels. Whether this regulation is beneficial or detrimental to the host is currently unknown and deserves further investigation.

References

1. Chan, J., and J. Flynn. 2004. The immunological aspects of latency in tuberculosis. *Clin Immunol.* 110: 2-12.
2. Lammas, D. A., J. L. Casanova, and D. S. Kumararatne. 2000. Clinical consequences of defects in the IL-12-dependent interferon-gamma (IFN-gamma) pathway. *Clin. Exp. Immunol.* 121: 417-425.
3. Lammas, D. A., E. De Heer, J. D. Edgar, V. Novelli, A. Ben-Smith, R. Baretto, P. Drysdale, J. Binch, C. MacLennan, D. S. Kumararatne, et al. 2002. Heterogeneity in the granulomatous response to mycobacterial infection in patients with defined genetic mutations in the interleukin 12-dependent interferon-gamma production pathway. *Int J Exp Pathol.* 83: 1-20.
4. Kochi, A. 1991. Government intervention programs in HIV/tuberculous infection. Outline of guidelines for national tuberculosis control programs in view of the HIV epidemic. *Bull Int Union Tuberc Lung Dis.* 66: 33-36.
5. Rook, G. A., K. Dheda, and A. Zumla. 2005. Opinion: Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nat. Rev. Immunol.* 5: 661-667.
6. Dheda, K., J. S. Chang, R. A. Breen, L. U. Kim, J. A. Haddock, J. F. Huggett, M. A. Johnson, G. A. Rook, and A. Zumla. 2005. In vivo and in vitro studies of a novel cytokine, interleukin 4delta2, in pulmonary tuberculosis. *Am. J. Respir. Crit. Care. Med.* 172: 501-508.
7. Ribeiro-Rodrigues, R., T. Resende Co, J. L. Johnson, F. Ribeiro, M. Palaci, R. T. Sa, E. L. Maciel, F. E. Pereira Lima, V. Dettoni, Z. Toossi, et al. 2002. Sputum cytokine levels in patients with pulmonary tuberculosis as early markers of mycobacterial clearance. *Clin Diagn Lab Immunol.* 9: 818-823.
8. Wilkinson, K. A., R. J. Wilkinson, A. Pathan, K. Ewer, M. Prakash, P. Klenerman, N. Maskell, R. Davies, G. Pasvol, and A. Lalvani. 2005. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. *Clin Infect Dis.* 40: 184-187.
9. Rook, G. A., J. Taverne, C. Leveton, and J. Steele. 1987. The role of gamma-interferon, vitamin D3 metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology.* 62: 229-234.
10. Rook, G. A. 1990. Mycobacteria, cytokines and antibiotics. *Pathol Biol (Paris).* 38: 276-280.
11. Hernandez-Pando, R., and G. A. Rook. 1994. The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology.* 82: 591-595.
12. al Attiyah, R., C. Moreno, and G. A. Rook. 1992. TNF alpha-mediated tissue damage in mouse footpads primed with mycobacterial preparations. *Res Immunol.* 143: 601-610.
13. Seah, G. T., and G. A. Rook. 2001. High levels of mRNA encoding IL-4 in unstimulated peripheral blood mononuclear cells from tuberculosis patients revealed by quantitative nested reverse transcriptase-polymerase chain reaction; correlations with serum IgE

levels. *Scand J Infect Dis.* 33: 106-109.

14. Rajavelu, P., and S. D. Das. 2005. Th2-type immune response observed in healthy individuals to sonicate antigen prepared from the most prevalent Mycobacterium tuberculosis strain with single copy of IS6110. *FEMS Immunol Med Microbiol.* 45: 95-102.
15. Lin, Y., M. Zhang, F. M. Hofman, J. Gong, and P. F. Barnes. 1996. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun.* 64: 1351-1356.
16. Rook, G. A., R. Hernandez-Pando, K. Dheda, and G. Teng Seah. 2004. IL-4 in tuberculosis: implications for vaccine design. *Trends Immunol.* 25: 483-488.
17. Rook, G. A., K. Dheda, and A. Zumla. 2005. Do successful tuberculosis vaccines need to be immunoregulatory rather than merely Th1-boosting? *Vaccine.* 23: 2115-2120.
18. Dheda, K., H. Booth, J. F. Huggett, M. A. Johnson, A. Zumla, and G. A. Rook. 2005. Lung Remodeling in Pulmonary Tuberculosis. *J Infect Dis.* 192: 1201-1209.
19. Aziz, M. A., and A. Wright. 2005. The World Health Organization/International Union Against Tuberculosis and Lung Disease Global Project on Surveillance for Anti-Tuberculosis Drug Resistance: a model for other infectious diseases. *Clin Infect Dis.* 41 Suppl 4: S258-262.
20. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature.* 388: 394-397.
21. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1-14.
22. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11: 443-451.
23. Takeuchi, O., T. Kawai, P. F. Muhlradt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13: 933-940.
24. Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169: 10-14.
25. Pasare, C., and R. Medzhitov. 2004. Toll-like receptors: linking innate and adaptive immunity. *Microbes. Infect.* 6: 1382-1387.
26. Pasare, C., and R. Medzhitov. 2005. Toll-like receptors: linking innate and adaptive immunity. *Adv. Exp. Med. Biol.* 560: 11-18.
27. Kang, T. J., S. B. Lee, and G. T. Chae. 2002. A polymorphism in the toll-like receptor 2 is associated with IL-12 production from monocyte in lepromatous leprosy. *Cytokine.* 20: 56-62.
28. Arbour, N. C., E. Lorenz, B. C. Schutte, J. Zabner, J. N. Kline, M. Jones, K. Frees, J. L. Watt, and D. A. Schwartz. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet.* 25: 187-191.
29. Abel, B., N. Thieblemont, V. J. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl, and B.

Ryffel. 2002. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J. Immunol.* 169: 3155-3162.

30. Sugawara, I., H. Yamada, C. Li, S. Mizuno, O. Takeuchi, and S. Akira. 2003. Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol. Immunol.* 47: 327-336.
31. Lorenz, E., J. P. Mira, K. L. Cornish, N. C. Arbour, and D. A. Schwartz. 2000. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect. Immun.* 68: 6398-6401.
32. Drennan, M. B., D. Nicolle, V. J. Quesniaux, M. Jacobs, N. Allie, J. Mpagi, C. Fremond, H. Wagner, C. Kirschning, and B. Ryffel. 2004. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am. J. Pathol.* 164: 49-57.
33. Krutzik, S. R., and R. L. Modlin. 2004. The role of Toll-like receptors in combating mycobacteria. *Semin Immunol.* 16: 35-41.
34. Tobian, A. A., N. S. Potter, L. Ramachandra, R. K. Pai, M. Convery, W. H. Boom, and C. V. Harding. 2003. Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen-associated molecular patterns: Mycobacterium tuberculosis 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. *J Immunol.* 171: 1413-1422.
35. Geijtenbeek, T. B., S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197: 7-17.
36. Gehring, A. J., K. M. Dobos, J. T. Belisle, C. V. Harding, and W. H. Boom. 2004. Mycobacterium tuberculosis LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J. Immunol.* 173: 2660-2668.
37. Dabbagh, K., M. E. Dahl, P. Stepick-Biek, and D. B. Lewis. 2002. Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. *J Immunol.* 168: 4524-4530.
38. Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med.* 196: 1645-1651.
39. Redecke, V., H. Hacker, S. K. Datta, A. Fermin, P. M. Pitha, D. H. Broide, and E. Raz. 2004. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J. Immunol.* 172: 2739-2743.
40. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol.* 171: 4984-4989.
41. Nigou, J., C. Zelle-Rieser, M. Gilleron, M. Thurnher, and G. Puzo. 2001. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol.* 166: 7477-7485.
42. Dabbagh, K., and D. B. Lewis. 2003. Toll-like receptors and T-helper-1/T-helper-2

responses. *Curr Opin Infect Dis.* 16: 199-204.

43. Krutzik, S. R., M. T. Ochoa, P. A. Sieling, S. Uematsu, Y. W. Ng, A. Legaspi, P. T. Liu, S. T. Cole, P. J. Godowski, Y. Maeda, et al. 2003. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat. Med.* 9: 525-532.
44. Fenhalls, G., G. R. Squires, L. Stevens-Muller, J. Bezuidenhout, G. Amphlett, K. Duncan, and P. T. Lukey. 2003. Associations between toll-like receptors and interleukin-4 in the lungs of patients with tuberculosis. *Am. J. Respir. Cell. Mol. Biol.* 29: 28-38.
45. Dheda, K., J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook, and A. Zumla. 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques.* 37: 112-114, 116, 118-119.
46. Dheda, K., J. F. Huggett, J. S. Chang, L. U. Kim, S. A. Bustin, M. A. Johnson, G. A. Rook, and A. Zumla. 2005. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal. Biochem.* 344: 141-143.
47. Boddinghaus, B., J. Wolters, W. Heikens, and E. C. Bottger. 1990. Phylogenetic analysis and identification of different serovars of Mycobacterium intracellulare at the molecular level. *FEMS Microbiol Lett.* 58: 197-203.
48. Frothingham, R., P. L. Strickland, G. Bretzel, S. Ramaswamy, J. M. Musser, and D. L. Williams. 1999. Phenotypic and genotypic characterization of Mycobacterium africanum isolates from West Africa. *J Clin Microbiol.* 37: 1921-1926.
49. Frothingham, R. 1999. Evolutionary bottlenecks in the agents of tuberculosis, leprosy, and paratuberculosis. *Med Hypotheses.* 52: 95-99.
50. Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford. 1990. Polymerase chain reaction amplification of a repetitive DNA sequence specific for Mycobacterium tuberculosis. *J Infect Dis.* 161: 977-981.
51. Gutacker, M. M., J. C. Smoot, C. A. Migliaccio, S. M. Ricklefs, S. Hua, D. V. Cousins, E. A. Graviss, E. Shashkina, B. N. Kreiswirth, and J. M. Musser. 2002. Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics.* 162: 1533-1543.
52. Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser. 1997. Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci U S A.* 94: 9869-9874.
53. Stead, W. W., K. D. Eisenach, M. D. Cave, M. L. Beggs, G. L. Templeton, C. O. Thoen, and J. H. Bates. 1995. When did Mycobacterium tuberculosis infection first occur in the New World? An important question with public health implications. *Am J Respir Crit Care Med.* 151: 1267-1268.
54. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature.* 393:

537-544.

55. Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*. 284: 1520-1523.
56. Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, et al. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. *Proc Natl Acad Sci U S A*. 99: 3684-3689.
57. Gutierrez, M. C., S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply, and V. Vincent. 2005. Ancient origin and gene mosaicism of the progenitor of mycobacteriumtuberculosis. *PLoS Pathog*. 1: e5.
58. Grange, J. M., and A. Zumla. 2002. The global emergency of tuberculosis: what is the cause? *J R Soc Health*. 122: 78-81.
59. Wang, P. D. 2002. Epidemiology and control of tuberculosis in Taipei. *J Infect*. 45: 82-87.
60. Maguire, H., J. W. Dale, T. D. McHugh, P. D. Butcher, S. H. Gillespie, A. Costetsos, H. Al-Ghusein, R. Holland, A. Dickens, L. Marston, et al. 2002. Molecular epidemiology of tuberculosis in London 1995-7 showing low rate of active transmission. *Thorax*. 57: 617-622.
61. Boudville, I. C., S. Y. Wong, and I. Snodgrass. 1997. Drug-resistant tuberculosis in Singapore, 1995 to 1996. *Ann Acad Med Singapore*. 26: 549-556.
62. Chan-Yeung, M., K. Noertjojo, C. C. Leung, S. L. Chan, and C. M. Tam. 2003. Prevalence and predictors of default from tuberculosis treatment in Hong Kong. *Hong Kong Med J*. 9: 263-268.
63. Hamada, M., K. Urabe, Y. Moroi, M. Miyazaki, and M. Furue. 2004. Epidemiology of cutaneous tuberculosis in Japan: a retrospective study from 1906 to 2002. *Int J Dermatol*. 43: 727-731.
64. Liu, C. E., C. H. Chen, J. H. Hsiao, T. G. Young, R. W. Tsay, and C. P. Fung. 2004. Drug resistance of Mycobacterium tuberculosis complex in central Taiwan. *J Microbiol Immunol Infect*. 37: 295-300.
65. Liaw, Y. S., P. R. Hsueh, C. J. Yu, S. K. Wang, P. C. Yang, and K. T. Luh. 2004. Drug resistance pattern of Mycobacterium tuberculosis in a university hospital in Taiwan, 1998-2002. *J Formos Med Assoc*. 103: 671-677.
66. Tsao, T. C., W. Chiou, H. Lin, T. Wu, M. Lin, P. Yang, and Y. Tsai. 2002. Change in demographic picture and increase of drug resistance in pulmonarytuberculosis in a 10-year interval in Taiwan. *Infection*. 30: 75-80.
67. Nakatani, H., N. Fujii, T. Mori, and H. Hoshinot. 2002. Epidemiological transition of tuberculosis and future agenda of control in Japan: results of the Ad-Hoc National Survey of Tuberculosis 2000. *Int J Tuberc Lung Dis*. 6: 198-207.
68. Liu, J. J., H. Y. Yao, and E. Y. Liu. 2005. Analysis of factors affecting the epidemiology of tuberculosis in China. *Int J Tuberc Lung Dis*. 9: 450-454.
69. Lin, Y. S., Y. C. Huang, L. Y. Chang, T. Y. Lin, and K. S. Wong. 2005. Clinical characteristics

of tuberculosis in children in the north of Taiwan. *J Microbiol Immunol Infect.* 38: 41-46.

70. Espinal, M. A., A. Laszlo, L. Simonsen, F. Boulahbal, S. J. Kim, A. Reniero, S. Hoffner, H. L. Rieder, N. Binkin, C. Dye, et al. 2001. Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. *N Engl J Med.* 344: 1294-1303.
71. Tang, S., and S. B. Squire. 2005. What lessons can be drawn from tuberculosis (TB) control in China in the 1990s? An analysis from a health system perspective. *Health Policy.* 72: 93-104.
72. Dannenberg, A. M., Jr. 1993. Immunopathogenesis of pulmonary tuberculosis. *Hosp Pract (Off Ed).* 28: 51-58.
73. Rook, G. A., G. Seah, and A. Ustianowski. 2001. M. tuberculosis: immunology and vaccination. *Eur Respir J.* 17: 537-557.
74. Kochi, A. 1991. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle.* 72: 1-6.
75. Hernandez-Pando, R., M. Jeyanathan, G. Mengistu, D. Aguilar, H. Orozco, M. Harboe, G. A. Rook, and G. Bjune. 2000. Persistence of DNA from Mycobacterium tuberculosis in superficially normal lung tissue during latent infection. *Lancet.* 356: 2133-2138.
76. Pan, H., B. S. Yan, M. Rojas, Y. V. Shebzukhov, H. Zhou, L. Kobzik, D. E. Higgins, M. J. Daly, B. R. Bloom, and I. Kramnik. 2005. Ipr1 gene mediates innate immunity to tuberculosis. *Nature.* 434: 767-772.
77. Grzybowski, S., and E. A. Allen. 1964. The Challenge of Tuberculosis in Decline. A Study Based on the Epidemiology of Tuberculosis in Ontario, Canada. *Am Rev Respir Dis.* 90: 707-720.
78. Stead, W. W. 1967. Pathogenesis of the sporadic case of tuberculosis. *N Engl J Med.* 277: 1008-1012.
79. Burgos, M. V., and A. S. Pym. 2002. Molecular epidemiology of tuberculosis. *Eur Respir J Suppl.* 36: 54s-65s.
80. Thierry, D., A. Brisson-Noel, V. Vincent-Levy-Frebault, S. Nguyen, J. L. Guesdon, and B. Gicquel. 1990. Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. *J Clin Microbiol.* 28: 2668-2673.
81. Van Soolingen, D. 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. *J Intern Med.* 249: 1-26.
82. Hernandez-Garduno, E., D. Kunimoto, L. Wang, M. Rodrigues, R. K. Elwood, W. Black, S. Mak, and J. M. FitzGerald. 2002. Predictors of clustering of tuberculosis in Greater Vancouver: a molecular epidemiologic study. *Cmaj.* 167: 349-352.
83. Geng, E., B. Kreiswirth, C. Driver, J. Li, J. Burzynski, P. DellaLatta, A. LaPaz, and N. W. Schluger. 2002. Changes in the transmission of tuberculosis in New York City from 1990 to 1999. *N Engl J Med.* 346: 1453-1458.
84. van Soolingen, D., M. W. Borgdorff, P. E. de Haas, M. M. Sebek, J. Veen, M. Dessens, K.

Kremer, and J. D. van Embden. 1999. Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. *J Infect Dis.* 180: 726-736.

85. Lillebaek, T., A. B. Andersen, J. Bauer, A. Dirksen, S. Glismann, P. de Haas, and A. Kok-Jensen. 2001. Risk of Mycobacterium tuberculosis transmission in a low-incidence country due to immigration from high-incidence areas. *J Clin Microbiol.* 39: 855-861.
86. Bauer, J., Z. Yang, S. Poulsen, and A. B. Andersen. 1998. Results from 5 years of nationwide DNA fingerprinting of Mycobacterium tuberculosis complex isolates in a country with a low incidence of M. tuberculosis infection. *J Clin Microbiol.* 36: 305-308.
87. van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden. 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *N Engl J Med.* 341: 1174-1179.
88. Sonnenberg, P., J. Murray, J. R. Glynn, S. Shearer, B. Kambashi, and P. Godfrey-Faussett. 2001. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. *Lancet.* 358: 1687-1693.
89. de Boer, A. S., and D. van Soolingen. 2000. Recurrent tuberculosis due to exogenous reinfection. *N Engl J Med.* 342: 1050-1051.
90. Seidler, A., A. Nienhaus, and R. Diel. 2004. The transmission of tuberculosis in the light of new molecular biological approaches. *Occup Environ Med.* 61: 96-102.
91. 1974. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Third report. East African-British Medical Research Councils. *Lancet.* 2: 237-240.
92. 1972. Controlled clinical trial of short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. *Lancet.* 1: 1079-1085.
93. Mitchison, D. A. 1998. How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. *Int J Tuberc Lung Dis.* 2: 10-15.
94. Janeway, C. A., Jr. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today.* 13: 11-16.
95. Janeway, C. A., Jr., and R. Medzhitov. 1998. Introduction: the role of innate immunity in the adaptive immune response. *Semin Immunol.* 10: 349-350.
96. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol.* 20: 197-216.
97. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol.* 12: 991-1045.
98. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23-35.
99. Rook, G. A., J. Steele, M. Ainsworth, and B. R. Champion. 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. *Immunology.* 59: 333-338.
100. Salgame, P. 2005. Host innate and Th1 responses and the bacterial factors that control Mycobacterium tuberculosis infection. *Curr Opin Immunol.* 17: 374-380.

101. Hart, P. D., M. R. Young, A. H. Gordon, and K. H. Sullivan. 1987. Inhibition of phagosome-lysosome fusion in macrophages by certain mycobacteria can be explained by inhibition of lysosomal movements observed after phagocytosis. *J Exp Med.* 166: 933-946.

102. Sturgill-Koszycki, S., U. E. Schaible, and D. G. Russell. 1996. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *Embo J.* 15: 6960-6968.

103. Chan, E. D., J. Chan, and N. W. Schluger. 2001. What is the role of nitric oxide in murine and human host defense against tuberculosis?Current knowledge. *Am J Respir Cell Mol Biol.* 25: 606-612.

104. Chan, J., K. Tanaka, D. Carroll, J. Flynn, and B. R. Bloom. 1995. Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis. *Infect Immun.* 63: 736-740.

105. Flynn, J. L., C. A. Scanga, K. E. Tanaka, and J. Chan. 1998. Effects of aminoguanidine on latent murine tuberculosis. *J Immunol.* 160: 1796-1803.

106. Adams, L. B., M. C. Dinauer, D. E. Morgenstern, and J. L. Krahenbuhl. 1997. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. *Tuber Lung Dis.* 78: 237-246.

107. Scanga, C. A., V. P. Mohan, K. Yu, H. Joseph, K. Tanaka, J. Chan, and J. L. Flynn. 2000. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. *J Exp Med.* 192: 347-358.

108. Aston, C., W. N. Rom, A. T. Talbot, and J. Reibman. 1998. Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide. *Am J Respir Crit Care Med.* 157: 1943-1950.

109. Schon, T., G. Elmberger, Y. Negesse, R. H. Pando, T. Sundqvist, and S. Britton. 2004. Local production of nitric oxide in patients with tuberculosis. *Int J Tuberc Lung Dis.* 8: 1134-1137.

110. Bertholet, S., E. Tzeng, E. Felley-Bosco, and J. Mauel. 1999. Expression of the inducible NO synthase in human monocytic U937 cells allows high output nitric oxide production. *J Leukoc Biol.* 65: 50-58.

111. Weinberg, J. B., M. A. Misukonis, P. J. Shami, S. N. Mason, D. L. Sauls, W. A. Dittman, E. R. Wood, G. K. Smith, B. McDonald, K. E. Bachus, et al. 1995. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood.* 86: 1184-1195.

112. Nathan, C., and M. U. Shiloh. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A.* 97: 8841-8848.

113. Friedman, C. R., G. C. Quinn, B. N. Kreiswirth, D. C. Perlman, N. Salomon, N. Schluger, M.

Lutfey, J. Berger, N. Poltoratskaia, and L. W. Riley. 1997. Widespread dissemination of a drug-susceptible strain of Mycobacterium tuberculosis. *J Infect Dis.* 176: 478-484.

114. Rockett, K. A., R. Brookes, I. Udalova, V. Vidal, A. V. Hill, and D. Kwiatkowski. 1998. 1,25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of Mycobacterium tuberculosis in a human macrophage-like cell line. *Infect Immun.* 66: 5314-5321.

115. Sharma, S., M. Sharma, S. Roy, P. Kumar, and M. Bose. 2004. Mycobacterium tuberculosis induces high production of nitric oxide in coordination with production of tumour necrosis factor-alpha in patients with fresh active tuberculosis but not in MDR tuberculosis. *Immunol Cell Biol.* 82: 377-382.

116. Lau, Y. L., G. C. Chan, S. Y. Ha, Y. F. Hui, and K. Y. Yuen. 1998. The role of phagocytic respiratory burst in host defense against Mycobacterium tuberculosis. *Clin Infect Dis.* 26: 226-227.

117. MacMicking, J. D., G. A. Taylor, and J. D. McKinney. 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science.* 302: 654-659.

118. Reid, S. D., G. Penna, and L. Adorini. 2000. The control of T cell responses by dendritic cell subsets. *Curr Opin Immunol.* 12: 114-121.

119. Adams, V. C., J. R. Hunt, R. Martinelli, R. Palmer, G. A. Rook, and L. R. Brunet. 2004. Mycobacterium vaccae induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. *Eur J Immunol.* 34: 631-638.

120. Luft, T., M. Jefford, P. Luetjens, T. Toy, H. Hochrein, K. A. Masterman, C. Maliszewski, K. Shortman, J. Cebon, and E. Maraskovsky. 2002. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. *Blood.* 100: 1362-1372.

121. Tan, J. K., and H. C. O'Neill. 2005. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J Leukoc Biol.* 78: 319-324.

122. Kikuchi, K., Y. Yanagawa, T. Aranami, C. Iwabuchi, K. Iwabuchi, and K. Onoe. 2003. Tumour necrosis factor-alpha but not lipopolysaccharide enhances preference of murine dendritic cells for Th2 differentiation. *Immunology.* 108: 42-49.

123. Sundquist, M., C. Johansson, and M. J. Wick. 2003. Dendritic cells as inducers of antimicrobial immunity in vivo. *Apmis.* 111: 715-724.

124. Hopken, U. E., I. Lehmann, J. Droese, M. Lipp, T. Schuler, and A. Rehm. 2005. The ratio between dendritic cells and T cells determines the outcome of their encounter: Proliferation versus deletion. *Eur J Immunol.*

125. Tailleux, L., O. Schwartz, J. L. Herrmann, E. Pivert, M. Jackson, A. Amara, L. Legres, D. Dreher, L. P. Nicod, J. C. Gluckman, et al. 2003. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *J. Exp. Med.* 197: 121-127.

126. Hertz, C. J., S. M. Kiertscher, P. J. Godowski, D. A. Bouis, M. V. Norgard, M. D. Roth, and R. L. Modlin. 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J Immunol.* 166: 2444-2450.

127. Tian, T., J. Woodworth, M. Skold, and S. M. Behar. 2005. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. *J Immunol.* 175: 3268-3272.

128. Henderson, R. A., S. C. Watkins, and J. L. Flynn. 1997. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol.* 159: 635-643.

129. Gonzalez-Juarrero, M., and I. M. Orme. 2001. Characterization of murine lung dendritic cells infected with Mycobacterium tuberculosis. *Infect Immun.* 69: 1127-1133.

130. Bodnar, K. A., N. V. Serbina, and J. L. Flynn. 2001. Fate of Mycobacterium tuberculosis within murine dendritic cells. *Infect Immun.* 69: 800-809.

131. Tailleux, L., O. Neyrolles, S. Honore-Bouakline, E. Perret, F. Sanchez, J. P. Abastado, P. H. Lagrange, J. C. Gluckman, M. Rosenzwajg, and J. L. Herrmann. 2003. Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells. *J Immunol.* 170: 1939-1948.

132. MacDonald, H. R., F. Radtke, and A. Wilson. 2001. T cell fate specification and alphabeta/gammadelta lineage commitment. *Curr Opin Immunol.* 13: 219-224.

133. MacDonald, H. R. 1995. NK1.1+ T cell receptor-alpha/beta+ cells: new clues to their origin, specificity, and function. *J Exp Med.* 182: 633-638.

134. Cowley, S. C., and K. L. Elkins. 2003. Multiple T cell subsets control Francisella tularensis LVS intracellular growth without stimulation through macrophage interferon gamma receptors. *J Exp Med.* 198: 379-389.

135. Cowley, S. C., E. Hamilton, J. A. Frelinger, J. Su, J. Forman, and K. L. Elkins. 2005. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. *J Exp Med.* 202: 309-319.

136. Flynn, J. L., and J. Chan. 2001. Immunology of tuberculosis. *Annu Rev Immunol.* 19: 93-129.

137. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med.* 178: 2243-2247.

138. Keane, J., S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med.* 345: 1098-1104.

139. Mohan, V. P., C. A. Scanga, K. Yu, H. M. Scott, K. E. Tanaka, E. Tsang, M. M. Tsai, J. L. Flynn, and J. Chan. 2001. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infect Immun.* 69: 1847-1855.

140. Flynn, J. L., and J. Chan. 2001. Tuberculosis: latency and reactivation. *Infect Immun.* 69: 4195-4201.

141. Lazarevic, V., D. Nolt, and J. L. Flynn. 2005. Long-term control of Mycobacterium tuberculosis infection is mediated by dynamic immune responses. *J Immunol.* 175: 1107-1117.

142. Kamath, A. B., J. Woodworth, X. Xiong, C. Taylor, Y. Weng, and S. M. Behar. 2004. Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after Mycobacterium tuberculosis infection. *J Exp Med.* 200: 1479-1489.

143. Serbina, N. V., C. C. Liu, C. A. Scanga, and J. L. Flynn. 2000. CD8+ CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. *J Immunol.* 165: 353-363.

144. Turner, J., C. D. D'Souza, J. E. Pearl, P. Marietta, M. Noel, A. A. Frank, R. Appelberg, I. M. Orme, and A. M. Cooper. 2001. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am J Respir Cell Mol Biol.* 24: 203-209.

145. Porcelli, S., M. B. Brenner, J. L. Greenstein, S. P. Balk, C. Terhorst, and P. A. Bleicher. 1989. Recognition of cluster of differentiation 1 antigens by human CD4-CD8-cytolytic T lymphocytes. *Nature.* 341: 447-450.

146. Porcelli, S. A. 1995. The CD1 family: a third lineage of antigen-presenting molecules. *Adv Immunol.* 59: 1-98.

147. Sieling, P. A. 2000. CD1-Restricted T cells: T cells with a unique immunological niche. *Clin Immunol.* 96: 3-10.

148. De Libero, G., and L. Mori. 2005. Recognition of lipid antigens by T cells. *Nat Rev Immunol.* 5: 485-496.

149. Brossay, L., D. Jullien, S. Cardell, B. C. Sydora, N. Burdin, R. L. Modlin, and M. Kronenberg. 1997. Mouse CD1 is mainly expressed on hemopoietic-derived cells. *J Immunol.* 159: 1216-1224.

150. Calabi, F., and A. Bradbury. 1991. The CD1 system. *Tissue Antigens.* 37: 1-9.

151. Sieling, P. A., M. T. Ochoa, D. Jullien, D. S. Leslie, S. Sabet, J. P. Rosat, A. E. Burdick, T. H. Rea, M. B. Brenner, S. A. Porcelli, et al. 2000. Evidence for human CD4+ T cells in the CD1-restricted repertoire: derivation of mycobacteria-reactive T cells from leprosy lesions. *J Immunol.* 164: 4790-4796.

152. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol.* 21: 483-513.

153. Van Rhijn, I., D. M. Zajonc, I. A. Wilson, and D. B. Moody. 2005. T-cell activation by lipopeptide antigens. *Curr Opin Immunol.* 17: 222-229.

154. Moody, D. B., T. Ulrichs, W. Muhlecker, D. C. Young, S. S. Gurcha, E. Grant, J. P. Rosat, M. B. Brenner, C. E. Costello, G. S. Besra, et al. 2000. CD1c-mediated T-cell recognition of isoprenoid glycolipids in Mycobacterium tuberculosis infection. *Nature.* 404: 884-888.

155. Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J. P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, et al. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science.* 276: 1684-1687.

156. Ulrichs, T., D. B. Moody, E. Grant, S. H. Kaufmann, and S. A. Porcelli. 2003. T-cell responses to CD1-presented lipid antigens in humans with Mycobacterium tuberculosis

infection. *Infect Immun.* 71: 3076-3087.

157. Smiley, S. T., M. H. Kaplan, and M. J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science.* 275: 977-979.

158. Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity.* 6: 459-467.

159. Modlin, R. L., and P. A. Sieling. 2005. Immunology. Now presenting: gammadelta T cells. *Science.* 309: 252-253.

160. Morita, C. T., R. A. Mariuzza, and M. B. Brenner. 2000. Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. *Springer Semin Immunopathol.* 22: 191-217.

161. Hayday, A. C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol.* 18: 975-1026.

162. Brandes, M., K. Willimann, and B. Moser. 2005. Professional antigen-presentation function by human gammadelta T Cells. *Science.* 309: 264-268.

163. Das, H., M. Sugita, and M. B. Brenner. 2004. Mechanisms of Vdelta1 gammadelta T cell activation by microbial components. *J Immunol.* 172: 6578-6586.

164. Morita, C. T., E. M. Beckman, J. F. Bukowski, Y. Tanaka, H. Band, B. R. Bloom, D. E. Golan, and M. B. Brenner. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity.* 3: 495-507.

165. Shin, S., R. El-Diwany, S. Schaffert, E. J. Adams, K. C. Garcia, P. Pereira, and Y. H. Chien. 2005. Antigen recognition determinants of gammadelta T cell receptors. *Science.* 308: 252-255.

166. Holtmeier, W., and D. Kabelitz. 2005. gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy.* 86: 151-183.

167. Boom, W. H. 1999. Gammadelta T cells and Mycobacterium tuberculosis. *Microbes Infect.* 1: 187-195.

168. Chen, Z. W. 2005. Immune regulation of gammadelta T cell responses in mycobacterial infections. *Clin Immunol.* 116: 202-207.

169. Janis, E. M., S. H. Kaufmann, R. H. Schwartz, and D. M. Pardoll. 1989. Activation of gamma delta T cells in the primary immune response to Mycobacterium tuberculosis. *Science.* 244: 713-716.

170. Barnes, P. F., C. L. Grisso, J. S. Abrams, H. Band, T. H. Rea, and R. L. Modlin. 1992. Gamma delta T lymphocytes in human tuberculosis. *J Infect Dis.* 165: 506-512.

171. Li, B., M. D. Rossman, T. Imir, A. F. Oner-Eyuboglu, C. W. Lee, R. Biancaniello, and S. R. Carding. 1996. Disease-specific changes in gammadelta T cell repertoire and function in patients with pulmonary tuberculosis. *J Immunol.* 157: 4222-4229.

172. Gioia, C., C. Agrati, R. Casetti, C. Cairo, G. Borsellino, L. Battistini, G. Mancino, D. Goletti, V. Colizzi, L. P. Pucillo, et al. 2002. Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. *J Immunol.* 168: 1484-1489.

173. Munk, M. E., A. J. Gatrill, and S. H. Kaufmann. 1990. Target cell lysis and IL-2 secretion by gamma/delta T lymphocytes after activation with bacteria. *J Immunol.* 145: 2434-2439.

174. Tsukaguchi, K., K. N. Balaji, and W. H. Boom. 1995. CD4+ alpha beta T cell and gamma delta T cell responses to Mycobacterium tuberculosis. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production. *J Immunol.* 154: 1786-1796.

175. Angelini, D. F., G. Borsellino, M. Poupot, A. Diamantini, R. Poupot, G. Bernardi, F. Poccia, J. J. Fournie, and L. Battistini. 2004. FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways. *Blood.* 104: 1801-1807.

176. Kaufmann, S. H. 2001. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol.* 1: 20-30.

177. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science.* 260: 547-549.

178. Keane, J., S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N. Engl. J. Med.* 345: 1098-1104.

179. Schaible, U. E., F. Winau, P. A. Sieling, K. Fischer, H. L. Collins, K. Hagens, R. L. Modlin, V. Brinkmann, and S. H. Kaufmann. 2003. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med.* 9: 1039-1046.

180. Hashimoto, C., K. L. Hudson, and K. V. Anderson. 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell.* 52: 269-279.

181. Nomura, N., N. Miyajima, T. Sazuka, A. Tanaka, Y. Kawarabayasi, S. Sato, T. Nagase, N. Seki, K. Ishikawa, and S. Tabata. 1994. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA Res.* 1: 27-35.

182. Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell.* 86: 973-983.

183. Beutler, B., K. Hoebe, P. Georgel, K. Tabeta, and X. Du. 2005. Genetic analysis of innate immunity: identification and function of the TIR adapter proteins. *Adv Exp Med Biol.* 560: 29-39.

184. Tauszig, S., E. Jouanguy, J. A. Hoffmann, and J. L. Imler. 2000. Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *Proc Natl Acad Sci U S A.* 97: 10520-10525.

185. Gay, N. J., and F. J. Keith. 1991. Drosophila Toll and IL-1 receptor. *Nature.* 351: 355-356.

186. Dunne, A., and L. A. O'Neill. 2003. The interleukin-1 receptor/Toll-like receptor superfamily:

signal transduction during inflammation and host defense. *Sci STKE*. 2003: re3.

187. Heguy, A., C. T. Baldari, G. Macchia, J. L. Telford, and M. Melli. 1992. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila toll protein are essential for IL-1R signal transduction. *J Biol Chem*. 267: 2605-2609.

188. Xu, Y., X. Tao, B. Shen, T. Horng, R. Medzhitov, J. L. Manley, and L. Tong. 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature*. 408: 111-115.

189. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 282: 2085-2088.

190. Mitsuzawa, H., I. Wada, H. Sano, D. Iwaki, S. Murakami, T. Himi, N. Matsushima, and Y. Kuroki. 2001. Extracellular Toll-like receptor 2 region containing Ser40-Ile64 but not Cys30-Ser39 is critical for the recognition of Staphylococcus aureus peptidoglycan. *J Biol Chem*. 276: 41350-41356.

191. Fujita, M., T. Into, M. Yasuda, T. Okusawa, S. Hamahira, Y. Kuroki, A. Eto, T. Nisizawa, M. Morita, and K. Shibata. 2003. Involvement of leucine residues at positions 107, 112, and 115 in a leucine-rich repeat motif of human Toll-like receptor 2 in the recognition of diacylated lipoproteins and lipopeptides and Staphylococcus aureus peptidoglycans. *J Immunol*. 171: 3675-3683.

192. Meng, G., A. Grabiec, M. Vallon, B. Ebe, S. Hampel, W. Bessler, H. Wagner, and C. J. Kirschning. 2003. Cellular recognition of tri-/di-palmitoylated peptides is independent from a domain encompassing the N-terminal seven leucine-rich repeat (LRR)/LRR-like motifs of TLR2. *J Biol Chem*. 278: 39822-39829.

193. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol*. 21: 335-376.

194. Takeuchi, O., T. Kawai, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Takeda, and S. Akira. 1999. TLR6: A novel member of an expanding toll-like receptor family. *Gene*. 231: 59-65.

195. Chuang, T. H., and R. J. Ulevitch. 2000. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw*. 11: 372-378.

196. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, et al. 2001. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature*. 413: 78-83.

197. Horng, T., G. M. Barton, and R. Medzhitov. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol*. 2: 835-841.

198. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol*. 169: 6668-6672.

199. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K.

Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol.* 4: 1144-1150.

200. Muzio, M., D. Bosisio, N. Polentarutti, G. D'Amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena, et al. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol.* 164: 5998-6004.
201. Zarember, K. A., and P. J. Godowski. 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol.* 168: 554-561.
202. Ochoa, M. T., A. J. Legaspi, Z. Hatziris, P. J. Godowski, R. L. Modlin, and P. A. Sieling. 2003. Distribution of Toll-like receptor 1 and Toll-like receptor 2 in human lymphoid tissue. *Immunology.* 108: 10-15.
203. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol.* 168: 4531-4537.
204. Matsumoto, M., K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, and T. Seya. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol.* 171: 3154-3162.
205. Ito, T., Y. H. Wang, and Y. J. Liu. 2005. Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. *Springer Semin Immunopathol.* 26: 221-229.
206. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med.* 197: 403-411.
207. Gao, B., and M. F. Tsan. 2003. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J Biol Chem.* 278: 174-179.
208. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature.* 413: 732-738.
209. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* 410: 1099-1103.
210. Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol.* 3: 196-200.
211. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.* 303: 1526-1529.

212. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*. 303: 1529-1531.

213. Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A*. 101: 5598-5603.

214. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, et al. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*. 408: 740-745.

215. Quesniaux, V., C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, et al. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect*. 6: 946-959.

216. Smith, M. F., Jr., A. Mitchell, G. Li, S. Ding, A. M. Fitzmaurice, K. Ryan, S. Crowe, and J. B. Goldberg. 2003. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem*. 278: 32552-32560.

217. Werts, C., R. I. Tapping, J. C. Mathison, T. H. Chuang, V. Kravchenko, I. Saint Girons, D. A. Haake, P. J. Godowski, F. Hayashi, A. Ozinsky, et al. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol*. 2: 346-352.

218. Sandor, F., E. Latz, F. Re, L. Mandell, G. Repik, D. T. Golenbock, T. Espevik, E. A. Kurt-Jones, and R. W. Finberg. 2003. Importance of extra- and intracellular domains of TLR1 and TLR2 in NFkappa B signaling. *J. Cell. Biol.* 162: 1099-1110.

219. Bulut, Y., E. Faure, L. Thomas, O. Equils, and M. Arditi. 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J. Immunol.* 167: 987-994.

220. Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.* 69: 1036-1044.

221. Means, T. K., B. W. Jones, A. B. Schromm, B. A. Shurtleff, J. A. Smith, J. Keane, D. T. Golenbock, S. N. Vogel, and M. J. Fenton. 2001. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. *J. Immunol.* 166: 4074-4082.

222. Wyllie, D. H., E. Kiss-Toth, A. Visintin, S. C. Smith, S. Boussouf, D. M. Segal, G. W. Duff, and S. K. Dower. 2000. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. *J. Immunol.* 165: 7125-7132.

223. Means, T. K., E. Lien, A. Yoshimura, S. Wang, D. T. Golenbock, and M. J. Fenton. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol.* 163: 6748-6755.

224. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. U S A.* 97: 13766-13771.

225. Ozinsky, A., K. D. Smith, D. Hume, and D. M. Underhill. 2000. Co-operative induction of pro-inflammatory signaling by Toll-like receptors. *J. Endotoxin. Res.* 6: 393-396.

226. Alexopoulou, L., V. Thomas, M. Schnare, Y. Lobet, J. Anguita, R. T. Schoen, R. Medzhitov, E. Fikrig, and R. A. Flavell. 2002. Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. *Nat. Med.* 8: 878-884.

227. Nakao, Y., K. Funami, S. Kikkawa, M. Taniguchi, M. Nishiguchi, Y. Fukumori, T. Seya, and M. Matsumoto. 2005. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *J. Immunol.* 174: 1566-1573.

228. Wang, P. Y., R. L. Kitchens, and R. S. Munford. 1995. Bacterial lipopolysaccharide binds to CD14 in low-density domains of the monocyte-macrophage plasma membrane. *J Inflamm.* 47: 126-137.

229. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* 387: 569-572.

230. Triantafilou, M., and K. Triantafilou. 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol.* 23: 301-304.

231. Triantafilou, M., S. Morath, A. Mackie, T. Hartung, and K. Triantafilou. 2004. Lateral diffusion of Toll-like receptors reveals that they are transiently confined within lipid rafts on the plasma membrane. *J Cell Sci.* 117: 4007-4014.

232. Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med.* 197: 1107-1117.

233. Doyle, S. E., R. M. O'Connell, G. A. Miranda, S. A. Vaidya, E. K. Chow, P. T. Liu, S. Suzuki, N. Suzuki, R. L. Modlin, W. C. Yeh, et al. 2004. Toll-like receptors induce a phagocytic gene program through p38. *J Exp Med.* 199: 81-90.

234. Chan, E. D., B. W. Winston, S. T. Uh, M. W. Wynes, D. M. Rose, and D. W. Riches. 1999. Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-gamma and TNF-alpha in mouse macrophages. *J Immunol.* 162: 415-422.

235. Adams, L. B., Y. Fukutomi, and J. L. Krahenbuhl. 1993. Regulation of murine macrophage effector functions by lipoarabinomannan from mycobacterial strains with different degrees of virulence. *Infect Immun.* 61: 4173-4181.

236. Chan, E. D., K. R. Morris, J. T. Belisle, P. Hill, L. K. Remigio, P. J. Brennan, and D. W. Riches. 2001. Induction of inducible nitric oxide synthase-NO* by lipoarabinomannan of Mycobacterium tuberculosis is mediated by MEK1-ERK, MKK7-JNK, and NF-kappaB signaling pathways. *Infect Immun.* 69: 2001-2010.

237. Tapping, R. I., and P. S. Tobias. 2003. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J Endotoxin Res.* 9: 264-268.

238. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science.* 285: 732-736.

239. Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, et al. 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science.* 291: 1544-1547.

240. Byrd-Leifer, C. A., E. F. Block, K. Takeda, S. Akira, and A. Ding. 2001. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. *Eur J Immunol.* 31: 2448-2457.

241. Kamath, A. B., J. Alt, H. Debbabi, and S. M. Behar. 2003. Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with Mycobacterium tuberculosis. *Infect. Immun.* 71: 4112-4118.

242. Fremond, C. M., V. Yeremeev, D. M. Nicolle, M. Jacobs, V. F. Quesniaux, and B. Ryffel. 2004. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J Clin Invest.* 114: 1790-1799.

243. Sugawara, I., H. Yamada, S. Mizuno, K. Takeda, and S. Akira. 2003. Mycobacterial infection in MyD88-deficient mice. *Microbiol Immunol.* 47: 841-847.

244. Jones, B. W., K. A. Heldwein, T. K. Means, J. J. Saukkonen, and M. J. Fenton. 2001. Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages. *Ann Rheum Dis.* 60 Suppl 3: iii6-12.

245. Lee, J. Y., C. A. Lowell, D. G. Lemay, H. S. Youn, S. H. Rhee, K. H. Sohn, B. Jang, J. Ye, J. H. Chung, and D. H. Hwang. 2005. The regulation of the expression of inducible nitric oxide synthase by Src-family tyrosine kinases mediated through MyD88-independent signaling pathways of Toll-like receptor 4. *Biochem Pharmacol.* 70: 1231-1240.

246. Reiling, N., C. Holscher, A. Fehrenbach, S. Kroger, C. J. Kirschning, S. Goyert, and S. Ehlers. 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol.* 169: 3480-3484.

247. Rehli, M. 2002. Of mice and men: species variations of Toll-like receptor expression. *Trends. Immunol.* 23: 375-378.

248. Lauener, R. P., T. Birchler, J. Adamski, C. Braun-Fahrlander, A. Bufe, U. Herz, E. von Mutius, D. Nowak, J. Riedler, M. Waser, et al. 2002. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet.* 360: 465-466.

249. Werner, M., R. Topp, K. Wimmer, K. Richter, W. Bischof, M. Wjst, and J. Heinrich. 2003. TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol.* 112: 323-330.

250. Bochud, P. Y., T. R. Hawn, and A. Aderem. 2003. Cutting edge: a Toll-like receptor 2

polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. *J. Immunol.* 170: 3451-3454.

251. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature.* 401: 811-815.
252. Blander, J. M., and R. Medzhitov. 2004. Regulation of phagosome maturation by signals from toll-like receptors. *Science.* 304: 1014-1018.
253. Chackerian, A. A., T. V. Perera, and S. M. Behar. 2001. Gamma interferon-producing CD4+ T lymphocytes in the lung correlate with resistance to infection with Mycobacterium tuberculosis. *Infect. Immun.* 69: 2666-2674.
254. Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani. 2003. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. *Lancet.* 361: 1168-1173.
255. Rainen, L., U. Oelmueller, S. Jurgensen, R. Wyrich, C. Ballas, J. Schram, C. Herdman, D. Bankaitis-Davis, N. Nicholls, D. Trollinger, et al. 2002. Stabilization of mRNA expression in whole blood samples. *Clin Chem.* 48: 1883-1890.
256. Rook, G. A., J. Steele, L. Fraher, S. Barker, R. Karmali, J. O'Riordan, and J. Stanford. 1986. Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes. *Immunology.* 57: 159-163.
257. Rook, G. A., and J. Steele. 1987. Macrophage regulation of vitamin D3 metabolites. *Nature.* 326: 21-22.
258. Kubin, M., J. M. Chow, and G. Trinchieri. 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, and IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood.* 83: 1847-1855.
259. Paul, R. C., J. L. Stanford, and J. W. Carswell. 1975. Multiple skin testing in leprosy. *J Hyg (Lond).* 75: 57-68.
260. Christian, W. a. 1941. *Biochem. Z.* 310: 384-421.
261. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-254.
262. Swartwout, S. G., and A. J. Kinniburgh. 1989. c-myc RNA degradation in growing and differentiating cells: possible alternate pathways. *Mol Cell Biol.* 9: 288-295.
263. Frevel, M. A., T. Bakheet, A. M. Silva, J. G. Hissong, K. S. Khabar, and B. R. Williams. 2003. p38 Mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. *Mol. Cell. Biol.* 23: 425-436.
264. Dheda, K., J. S. Chang, L. U. Kim, J. F. Huggett, M. A. Johnson, A. Zumla, and G. A. Rook. 2005. Interferon gamma assays for tuberculosis. *Lancet Infect Dis.* 5: 324-325.
265. Akashi, S., S. Saitoh, Y. Wakabayashi, T. Kikuchi, N. Takamura, Y. Nagai, Y. Kusumoto, K. Fukase, S. Kusumoto, Y. Adachi, et al. 2003. Lipopolysaccharide interaction with cell

surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J Exp Med.* 198: 1035-1042.

266. Rehli, M., A. Poltorak, L. Schwarzfischer, S. W. Krause, R. Andreesen, and B. Beutler. 2000. PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human Toll-like receptor 4 gene. *J. Biol. Chem.* 275: 9773-9781.
267. Komai-Koma, M., L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew. 2004. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci U S A.* 101: 3029-3034.
268. Iwami, K. I., T. Matsuguchi, A. Masuda, T. Kikuchi, T. Musikacharoen, and Y. Yoshikai. 2000. Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. *J. Immunol.* 165: 6682-6686.
269. LeBouder, E., J. E. Rey-Nores, N. K. Rushmere, M. Grigorov, S. D. Lawn, M. Affolter, G. E. Griffin, P. Ferrara, E. J. Schiffrin, B. P. Morgan, et al. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *J. Immunol.* 171: 6680-6689.
270. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science.* 230: 1350-1354.
271. Huggett, J., K. Dheda, S. Bustin, and A. Zumla. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes. Immun.* 6: 279-284.
272. Ullmannova, V., and C. Haskovec. 2003. The use of housekeeping genes (HKG) as an internal control for the detection of gene expression by quantitative real-time RT-PCR. *Folia Biol (Praha).* 49: 211-216.
273. Rhoads, R. P., C. McManaman, K. L. Ingvartsen, and Y. R. Boisclair. 2003. The housekeeping genes GAPDH and cyclophilin are regulated by metabolic state in the liver of dairy cows. *J Dairy Sci.* 86: 3423-3429.
274. Glare, E. M., M. Divjak, M. J. Bailey, and E. H. Walters. 2002. beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax.* 57: 765-770.
275. Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* 29: 23-39.
276. Barbu, V., and F. Dautry. 1989. Northern blot normalization with a 28S rRNA oligonucleotide probe. *Nucleic Acids Res.* 17: 7115.
277. Bustin S, N. T. 2004. Template handling, Preparation, and Quantification. In *A-Z of Quantitative PCR.* B. SA, ed. La Jolla, Ca: International University Line.
278. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 162: 156-159.
279. Tricarico, C., P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, S. A. Bustin, and C. Orlando. 2002. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for

human tissue biopsies. *Anal Biochem.* 309: 293-300.

280. Spanakis, E. 1993. Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. *Nucleic Acids Res.* 21: 3809-3819.

281. Raaijmakers, M. H., L. van Emst, T. de Witte, E. Mensink, and R. A. Raymakers. 2002. Quantitative assessment of gene expression in highly purified hematopoietic cells using real-time reverse transcriptase polymerase chain reaction. *Exp Hematol.* 30: 481-487.

282. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol.* 63: 3741-3751.

283. Tichopad, A., A. Didier, and M. W. Pfaffl. 2004. Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. *Mol Cell Probes.* 18: 45-50.

284. Izraeli, S., C. Pfleiderer, and T. Lion. 1991. Detection of gene expression by PCR amplification of RNA derived from frozen heparinized whole blood. *Nucleic Acids Res.* 19: 6051.

285. Raja, S., J. D. Luketich, L. A. Kelly, D. W. Ruff, and T. E. Godfrey. 2000. Increased sensitivity of one-tube, quantitative RT-PCR. *Biotechniques.* 29: 702, 704, 706.

286. Freeman, W. M., S. L. Vrana, and K. E. Vrana. 1996. Use of elevated reverse transcription reaction temperatures in RT-PCR. *Biotechniques.* 20: 782-783.

287. Freeman, W. M., S. J. Walker, and K. E. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques.* 26: 112-122, 124-115.

288. Bustin, S. A. 2005. Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. *Expert. Rev. Mol. Diagn.* 5: 493-498.

289. Jeffreys, A. J., V. Wilson, R. Neumann, and J. Keyte. 1988. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res.* 16: 10953-10971.

290. Karrer, E. E., J. E. Lincoln, S. Hogenhout, A. B. Bennett, R. M. Bostock, B. Martineau, W. J. Lucas, D. G. Gilchrist, and D. Alexander. 1995. In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. *Proc Natl Acad Sci U S A.* 92: 3814-3818.

291. Glasel, J. A. 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *Biotechniques.* 18: 62-63.

292. Jones, L. J., S. T. Yue, C. Y. Cheung, and V. L. Singer. 1998. RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. *Anal Biochem.* 265: 368-374.

293. Pasare, C., and R. Medzhitov. 2004. Toll-like receptors and acquired immunity. *Semin. Immunol.* 16: 23-26.

294. Ross, J. 1995. mRNA stability in mammalian cells. *Microbiol. Rev.* 59: 423-450.

295. Hansen, M. C., A. K. Nielsen, S. Molin, K. Hammer, and M. Kilstrup. 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. *J*

Bacteriol. 183: 4747-4751.

296. Pasare, C., and R. Medzhitov. 2004. Toll-dependent control mechanisms of CD4 T cell activation. *Immunity.* 21: 733-741.

297. Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J. Immunol.* 163: 3920-3927.

298. Branger, J., S. Knapp, S. Weijer, J. C. Leemans, J. M. Pater, P. Speelman, S. Florquin, and T. van der Poll. 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect. Immun.* 72: 788-794.

299. Wang, G., X. Guo, and J. Floros. 2005. Differences in the translation efficiency and mRNA stability mediated by 5'-UTR splice variants of human SP-A1 and SP-A2 genes. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 289: L497-508.

300. Pfeifer, I., C. Anderson, R. Werner, and E. Oltra. 2004. Redefining the structure of the mouse connexin43 gene: selective promoter usage and alternative splicing mechanisms yield transcripts with different translational efficiencies. *Nucleic. Acids. Res.* 32: 4550-4562.

301. van der Velden, A. W., and A. A. Thomas. 1999. The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int. J Biochem. Cell. Biol.* 31: 87-106.

302. Ito, T., R. Amakawa, and S. Fukuhara. 2002. Roles of toll-like receptors in natural interferon-producing cells as sensors in immune surveillance. *Hum Immunol.* 63: 1120-1125.

303. Mazzoni, A., and D. M. Segal. 2004. Controlling the Toll road to dendritic cell polarization. *J Leukoc Biol.* 75: 721-730.

304. Latchumanan, V. K., M. Y. Balkhi, A. Sinha, B. Singh, P. Sharma, and K. Natarajan. 2005. Regulation of immune responses to Mycobacterium tuberculosis secretory antigens by dendritic cells. *Tuberculosis (Edinb).*

305. Haehnel, V., L. Schwarzfischer, M. J. Fenton, and M. Rehli. 2002. Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. *J. Immunol.* 168: 5629-5637.

306. Fan, J., A. Kapus, P. A. Marsden, Y. H. Li, G. Oreopoulos, J. C. Marshall, S. Frantz, R. A. Kelly, R. Medzhitov, and O. D. Rotstein. 2002. Regulation of Toll-like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. *J. Immunol.* 168: 5252-5259.

307. Jorgensen, R. A., P. D. Cluster, J. English, Q. Que, and C. A. Napoli. 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. *Plant Mol Biol.* 31: 957-973.

308. Hamilton, A. J., and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science.* 286: 950-952.

309. Blaschke, E., A. Eklund, S. Skog, and B. Danielsson. 1985. Isolation of human alveolar macrophages and lymphocytes from bronchoalveolar lavage fluid by centrifugal elutriation. *Scand J Clin Lab Invest.* 45: 691-696.

310. Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan. 1998. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A.* 95: 588-593.

311. Stevenson, B. J., C. Iseli, B. Beutler, and C. V. Jongeneel. 2003. Use of transcriptome data to unravel the fine structure of genes involved in sepsis. *J Infect Dis.* 187 Suppl 2: S308-314.

312. Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias. 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity.* 10: 387-398.

313. Mavropoulos, A., G. Sully, A. P. Cope, and A. R. Clark. 2005. Stabilization of IFN-gamma mRNA by MAPK p38 in IL-12- and IL-18-stimulated human NK cells. *Blood.* 105: 282-288.

314. Modrek, B., A. Resch, C. Grasso, and C. Lee. 2001. Genome-wide detection of alternative splicing in expressed sequences of human genes. *Nucleic Acids Res.* 29: 2850-2859.

315. Jordan, T., S. Schornack, and T. Lahaye. 2002. Alternative splicing of transcripts encoding Toll-like plant resistance proteins - what's the functional relevance to innate immunity? *Trends Plant Sci.* 7: 392-398.

316. Haehnel, V., L. Schwarzfischer, M. J. Fenton, and M. Rehli. 2002. Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. *J Immunol.* 168: 5629-5637.

317. Lopez, A. J. 1998. Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. *Annu Rev Genet.* 32: 279-305.

318. Bopp, D., L. R. Bell, T. W. Cline, and P. Schedl. 1991. Developmental distribution of female-specific Sex-lethal proteins in Drosophila melanogaster. *Genes Dev.* 5: 403-415.

319. Mironov, A. A., J. W. Fickett, and M. S. Gelfand. 1999. Frequent alternative splicing of human genes. *Genome Res.* 9: 1288-1293.

320. Minegishi, N., J. Ohta, N. Suwabe, H. Nakauchi, H. Ishihara, N. Hayashi, and M. Yamamoto. 1998. Alternative promoters regulate transcription of the mouse GATA-2 gene. *J. Biol. Chem.* 273: 3625-3634.

321. Orban, T. I., and E. Olah. 2003. Emerging roles of BRCA1 alternative splicing. *Mol. Pathol.* 56: 191-197.

322. Tebo, J. M., S. Datta, R. Kishore, M. Kolosov, J. A. Major, Y. Ohmori, and T. A. Hamilton. 2000. Interleukin-1-mediated stabilization of mouse KC mRNA depends on sequences in both 5'- and 3'-untranslated regions. *J. Biol. Chem.* 275: 12987-12993.

323. Saklatvala, J. 2004. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr. Opin. Pharmacol.* 4: 372-377.

324. Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *Embo J.* 18: 4969-4980.

325. Brook, M., G. Sully, A. R. Clark, and J. Saklatvala. 2000. Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS. Lett.* 483: 57-61.

326. Laroux, F. S., X. Romero, L. Wetzler, P. Engel, and C. Terhorst. 2005. Cutting edge: MyD88 controls phagocyte NADPH oxidase function and killing of gram-negative bacteria. *J Immunol.* 175: 5596-5600.

327. Into, T., and K. Shibata. 2005. Apoptosis signal-regulating kinase 1-mediated sustained p38 mitogen-activated protein kinase activation regulates mycoplasmal lipoprotein- and staphylococcal peptidoglycan-triggered Toll-like receptor 2 signalling pathways. *Cell Microbiol.* 7: 1305-1317.

328. Lin, Y. L., Y. C. Liang, S. S. Lee, and B. L. Chiang. 2005. Polysaccharide purified from Ganoderma lucidum induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. *J Leukoc Biol.* 78: 533-543.

329. Maung, A. A., S. Fujimi, M. L. Miller, M. P. MacConmara, J. A. Mannick, and J. A. Lederer. 2005. Enhanced TLR4 reactivity following injury is mediated by increased p38 activation. *J Leukoc Biol.* 78: 565-573.

330. Sobell, H. M. 1985. Actinomycin and DNA transcription. *Proc Natl Acad Sci U S A.* 82: 5328-5331.

331. Egyhazi, E. 1976. Quantitation of turnover and export to the cytoplasm of hnRNA transcribed in the Balbiani rings. *Cell.* 7: 507-515.

332. de Mercoyrol, L., C. Job, and D. Job. 1989. Studies on the inhibition by alpha-amanitin of single-step addition reactions and productive RNA synthesis catalysed by wheat-germ RNA polymerase II. *Biochem J.* 258: 165-169.

333. Leclerc, G. J., G. M. Leclerc, and J. C. Barredo. 2002. Real-time RT-PCR analysis of mRNA decay: half-life of Beta-actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines. *Cancer Cell Int.* 2: 1.

334. Raghavan, A., R. L. Ogilvie, C. Reilly, M. L. Abelson, S. Raghavan, J. Vasdewani, M. Krathwohl, and P. R. Bohjanen. 2002. Genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes. *Nucleic Acids Res.* 30: 5529-5538.

335. Hargrove, J. L., and F. H. Schmidt. 1989. The role of mRNA and protein stability in gene expression. *Faseb J.* 3: 2360-2370.

336. Barrachina, M., E. Gonalons, and A. Celada. 1999. LPS upregulates MHC class II I-A expression in B lymphocytes at transcriptional and at translational levels. *Tissue. Antigens.* 54: 461-470.

337. Fitzgerald, M. L., K. J. Moore, M. W. Freeman, and G. L. Reed. 2000. Lipopolysaccharide

induces scavenger receptor A expression in mouse macrophages: a divergent response relative to human THP-1 monocyte/macrophages. *J Immunol.* 164: 2692-2700.

338. Ayoubi, T. A., and W. J. Van De Ven. 1996. Regulation of gene expression by alternative promoters. *Faseb J.* 10: 453-460.

339. Tolner, B., K. Roy, and F. M. Sirotnak. 1998. Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity. *Gene.* 211: 331-341.

340. Nakamuta, M., K. Oka, J. Krushkal, K. Kobayashi, M. Yamamoto, W. H. Li, and L. Chan. 1995. Alternative mRNA splicing and differential promoter utilization determine tissue-specific expression of the apolipoprotein B mRNA-editing protein (Apobec1) gene in mice. Structure and evolution of Apobec1 and related nucleoside/nucleotide deaminases. *J Biol Chem.* 270: 13042-13056.

341. Rajagopalan, S., D. F. Wan, G. M. Habib, A. R. Sepulveda, M. R. McLeod, R. M. Lebovitz, and M. W. Lieberman. 1993. Six mRNAs with different 5' ends are encoded by a single gamma-glutamyltransferase gene in mouse. *Proc Natl Acad Sci U S A.* 90: 6179-6183.

342. Schibler, U., and F. Sierra. 1987. Alternative promoters in developmental gene expression. *Annu Rev Genet.* 21: 237-257.

343. Patel, M., D. Xu, P. Kewin, B. Choo-Kang, C. McSharry, N. C. Thomson, and F. Y. Liew. 2005. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol.* 174: 7558-7563.

344. van der Kleij, D., E. Latz, J. F. Brouwers, Y. C. Kruize, M. Schmitz, E. A. Kurt-Jones, T. Espevik, E. C. de Jong, M. L. Kapsenberg, D. T. Golenbock, et al. 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem.* 277: 48122-48129.

345. Atamas, S. P., J. Choi, V. V. Yurovsky, and B. White. 1996. An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation. *J Immunol.* 156: 435-441.

346. Vasiliev, A. M., R. N. Vasilenko, N. L. Kulikova, S. M. Andreev, I. O. Chikileva, G. Y. Puchkova, I. V. Kosarev, A. V. Khodyakova, V. S. Khlebnikov, L. R. Ptitsyn, et al. 2003. Structural and functional properties of IL-4delta2, an alternative splice variant of human IL-4. *J Proteome Res.* 2: 273-281.

347. Demissie, A., M. Abebe, A. Aseffa, G. Rook, H. Fletcher, A. Zumla, K. Weldingh, I. Brock, P. Andersen, and T. M. Doherty. 2004. Healthy individuals that control a latent infection with Mycobacterium tuberculosis express high levels of Th1 cytokines and the IL-4 antagonist IL-4delta2. *J Immunol.* 172: 6938-6943.

348. Fletcher, H. A., P. Owiafe, D. Jeffries, P. Hill, G. A. Rook, A. Zumla, T. M. Doherty, and R. H. Brookes. 2004. Increased expression of mRNA encoding interleukin (IL)-4 and its splice variant IL-4delta2 in cells from contacts of Mycobacterium tuberculosis, in the absence of in vitro stimulation. *Immunology.* 112: 669-673.

349. Kaisho, T., K. Hoshino, T. Iwabe, O. Takeuchi, T. Yasui, and S. Akira. 2002. Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. *Int Immunol.* 14: 695-700.

350. Hajjar, A. M., D. S. O'Mahony, A. Ozinsky, D. M. Underhill, A. Aderem, S. J. Klebanoff, and C. B. Wilson. 2001. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J Immunol.* 166: 15-19.

351. Zhang, M., J. Gong, Z. Yang, B. Samten, M. D. Cave, and P. F. Barnes. 1999. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. *J Infect Dis.* 179: 1213-1217.

352. Bellamy, R., C. Ruwende, T. Corrah, K. P. McAdam, H. C. Whittle, and A. V. Hill. 1998. Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. *N Engl J Med.* 338: 640-644.

353. Gruenheid, S., E. Pinner, M. Desjardins, and P. Gros. 1997. Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *J Exp Med.* 185: 717-730.

354. Manca, C., L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J. M. Musser, C. E. Barry, 3rd, V. H. Freedman, and G. Kaplan. 2001. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. *Proc Natl Acad Sci U S A.* 98: 5752-5757.

355. Theus, S. A., M. D. Cave, and K. D. Eisenach. 2005. Intracellular macrophage growth rates and cytokine profiles of Mycobacterium tuberculosis strains with different transmission dynamics. *J Infect Dis.* 191: 453-460.

356. Manca, C., M. B. Reed, S. Freeman, B. Mathema, B. Kreiswirth, C. E. Barry, 3rd, and G. Kaplan. 2004. Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. *Infect Immun.* 72: 5511-5514.

357. Zuany-Amorim, C., C. Manlius, A. Trifilieff, L. R. Brunet, G. Rook, G. Bowen, G. Pay, and C. Walker. 2002. Long-term protective and antigen-specific effect of heat-killed Mycobacterium vaccae in a murine model of allergic pulmonary inflammation. *J Immunol.* 169: 1492-1499.

358. Zheng, X. M., S. M. Li, and B. C. Xing. 2004. [Short-term effect of treatment protocol utilizing levofloxacin, pasiniazide and M.Vaccae on multi- drug resistant pulmonary tuberculosis]. *Di Yi Jun Yi Da Xue Xue Bao.* 24: 574-575, 578.

359. Luo, Y., S. Lu, and S. Guo. 2000. [Immunotherapeutic effect of Mycobacterium vaccae on multi-drug resistant pulmonary tuberculosis]. *Zhonghua Jie He He Hu Xi Za Zhi.* 23: 85-88.

360. Wang, W., G. Jin, Y. Ye, X. Xia, A. Wang, Y. Zhuang, G. Li, H. Sun, Z. Wang, M. Lin, et al. 1999. [A clinical study on vaccine of Mycobacterium vaccae in treating pulmonary tuberculosis]. *Zhonghua Jie He He Hu Xi Za Zhi.* 22: 108-110.

361. Anderson, K. V. 2000. Toll signaling pathways in the innate immune response. *Curr Opin Immunol.* 12: 13-19.

362. Lodish H, B. A., Zipursky LS, Matsudaira P, Baltimore D, Darnell J. 2000. *Molecular cell biology*. WH Freeman and company.

363. Munder, M., K. Eichmann, and M. Modolell. 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. *J Immunol.* 160: 5347-5354.

364. Goerdt, S., and C. E. Orfanos. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity.* 10: 137-142.

365. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23: 549-555.

366. Brodin, P., I. Rosenkrands, P. Andersen, S. T. Cole, and R. Brosch. 2004. ESAT-6 proteins: protective antigens and virulence factors? *Trends Microbiol.* 12: 500-508.

367. Gey van Pittius, N. C., R. M. Warren, and P. D. van Helden. 2002. ESAT-6 and CFP-10: what is the diagnosis? *Infect Immun.* 70: 6509-6510; author reply 6511.

368. Ishii, K. J., and S. Akira. 2005. Innate immune recognition of nucleic acids: beyond toll-like receptors. *Int J Cancer.* 117: 517-523.

369. Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakanishi, M. Kimoto, K. Miyake, K. Takeda, et al. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol.* 164: 3476-3479.

370. Janssens, S., K. Burns, E. Vercammen, J. Tschopp, and R. Beyaert. 2003. MyD88S, a splice variant of MyD88, differentially modulates NF-kappaB- and AP-1-dependent gene expression. *FEBS. Lett.* 548: 103-107.

371. Wesche, H., X. Gao, X. Li, C. J. Kirschning, G. R. Stark, and Z. Cao. 1999. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. *J Biol Chem.* 274: 19403-19410.

372. Sun, J., F. Wiklund, S. L. Zheng, B. Chang, K. Balter, L. Li, J. E. Johansson, G. Li, H. O. Adami, W. Liu, et al. 2005. Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk. *J Natl Cancer Inst.* 97: 525-532.

373. Pirie, F. J., R. Pegoraro, A. A. Motala, S. Rauff, L. Rom, T. Govender, and T. M. Esterhuizen. 2005. Toll-like receptor 3 gene polymorphisms in South African Blacks with type 1 diabetes. *Tissue Antigens.* 66: 125-130.

374. Oostenbrug, L. E., J. P. Drenth, D. J. de Jong, I. M. Nolte, E. Oosterom, H. M. van Dullemen, K. van der Linde, G. J. te Meerman, G. van der Steege, J. H. Kleibeuker, et al. 2005. Association between Toll-like receptor 4 and inflammatory bowel disease. *Inflamm Bowel Dis.* 11: 567-575.

375. Fageras Bottcher, M., M. Hmani-Aifa, A. Lindstrom, M. C. Jenmalm, X. M. Mai, L. Nilsson, H. A. Zdolsek, B. Bjorksten, P. Soderkvist, and O. Vaarala. 2004. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70)

responses in Swedish children. *J Allergy Clin Immunol.* 114: 561-567.

376. Lazarus, R., B. A. Raby, C. Lange, E. K. Silverman, D. J. Kwiatkowski, D. Vercelli, W. J. Klimecki, F. D. Martinez, and S. T. Weiss. 2004. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med.* 170: 594-600.

377. Braun-Fahrlander, C., J. Riedler, U. Herz, W. Eder, M. Waser, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Bufe, et al. 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med.* 347: 869-877.

378. Eder, W., W. Klimecki, L. Yu, E. von Mutius, J. Riedler, C. Braun-Fahrlander, D. Nowak, and F. D. Martinez. 2004. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol.* 113: 482-488.

379. Noguchi, E., F. Nishimura, H. Fukai, J. Kim, K. Ichikawa, M. Shibasaki, and T. Arinami. 2004. An association study of asthma and total serum immunoglobin E levels for Toll-like receptor polymorphisms in a Japanese population. *Clin Exp Allergy.* 34: 177-183.

380. Raby, B. A., W. T. Klimecki, C. Laprise, Y. Renaud, J. Faith, M. Lemire, C. Greenwood, K. M. Weiland, C. Lange, L. J. Palmer, et al. 2002. Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. *Am J Respir Crit Care Med.* 166: 1449-1456.

381. Yang, I. A., S. J. Barton, S. Rorke, J. A. Cakebread, T. P. Keith, J. B. Clough, S. T. Holgate, and J. W. Holloway. 2004. Toll-like receptor 4 polymorphism and severity of atopy in asthmatics. *Genes Immun.* 5: 41-45.

382. Boonstra, A., C. Asselin-Paturel, M. Gilliet, C. Crain, G. Trinchieri, Y. J. Liu, and A. O'Garra. 2003. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med.* 197: 101-109.

383. Moseman, E. A., X. Liang, A. J. Dawson, A. Panoskaltsis-Mortari, A. M. Krieg, Y. J. Liu, B. R. Blazar, and W. Chen. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol.* 173: 4433-4442.

384. Hayashi, T., L. Beck, C. Rossetto, X. Gong, O. Takikawa, K. Takabayashi, D. H. Broide, D. A. Carson, and E. Raz. 2004. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J Clin Invest.* 114: 270-279.

385. Fanucchi, M. V., E. S. Schelegle, G. L. Baker, M. J. Evans, R. J. McDonald, L. J. Gershwin, E. Raz, D. M. Hyde, C. G. Plopper, and L. A. Miller. 2004. Immunostimulatory oligonucleotides attenuate airways remodeling in allergic monkeys. *Am J Respir Crit Care Med.* 170: 1153-1157.

386. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature.* 416: 603-607.

387. Christensen, S. R., M. Kashgarian, L. Alexopoulou, R. A. Flavell, S. Akira, and M. J. Shlomchik. 2005. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J Exp Med.* 202: 321-331.

388. Sano, K., H. Shirota, T. Terui, T. Hattori, and G. Tamura. 2003. Oligodeoxynucleotides without CpG motifs work as adjuvant for the induction of Th2 differentiation in a sequence-independent manner. *J Immunol.* 170: 2367-2373.

389. Vollmer, J., R. D. Weeratna, M. Jurk, U. Samulowitz, M. J. McCluskie, P. Payette, H. L. Davis, C. Schetter, and A. M. Krieg. 2004. Oligodeoxynucleotides lacking CpG dinucleotides mediate Toll-like receptor 9 dependent T helper type 2 biased immune stimulation. *Immunology.* 113: 212-223.

390. Ng, M. W., C. S. Lau, T. M. Chan, W. H. Wong, and Y. L. Lau. 2005. Polymorphisms of the toll-like receptor 9 (TLR9) gene with systemic lupus erythematosus in Chinese. *Rheumatology (Oxford).* 44: 1456-1457.

391. Hur, J. W., H. D. Shin, B. L. Park, L. H. Kim, S. Y. Kim, and S. C. Bae. 2005. Association study of Toll-like receptor 9 gene polymorphism in Korean patients with systemic lupus erythematosus. *Tissue Antigens.* 65: 266-270.

392. Zuany-Amorim, C., E. Sawicka, C. Manlius, A. Le Moine, L. R. Brunet, D. M. Kemeny, G. Bowen, G. Rook, and C. Walker. 2002. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. *Nat Med.* 8: 625-629.

393. Jang, S., S. Uematsu, S. Akira, and P. Salgame. 2004. IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition. *J Immunol.* 173: 3392-3397.

394. Re, F., and J. L. Strominger. 2004. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J Immunol.* 173: 7548-7555.

395. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 299: 1033-1036.

396. Frost, R. A., G. J. Nystrom, and C. H. Lang. 2005. Multiple Toll-like Receptor Ligands Induce an IL-6 Transcriptional Response in Skeletal Myoctyes. *Am J Physiol Regul Integr Comp Physiol.*

397. Girard, M. P., U. Fruth, and M. P. Kieny. 2005. A review of vaccine research and development: Tuberculosis. *Vaccine.* 23: 5725-5731.

Appendix

Appendix 1.1.

>gi|41350336|ref|NM_003263.3| Homo sapiens toll-like receptor 1 (TLR1), mRNA

Length=2867

Score = 5245 bits (2646), Expect = 0.0

Identities = 2646/2646 (100%), Gaps = 0/2646 (0%)

Strand=Plus/Plus

```
Query  38    GGGTCTTCATGAACACTAATAGGGGTACCAGGCCCTCTTCCTCGTTAGAAGAAATCAGGA  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115   GGGTCTTCATGAACACTAATAGGGGTACCAGGCCCTCTTCCTCGTTAGAAGAAATCAGGA  174

Query  98    TAACAAAGGCATATTGGGCACCCCTACAAAAGGAATCTGTATCTGTATCAAGATGATCTG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175   TAACAAAGGCATATTGGGCACCCCTACAAAAGGAATCTGTATCTGTATCAAGATGATCTG  234

Query  158   AAGAACAGCTTCTACCTTTAGGAATGTCTAGTGTTCCAAAATGACTAGCATCTTCCATTT  217
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235   AAGAACAGCTTCTACCTTTAGGAATGTCTAGTGTTCCAAAATGACTAGCATCTTCCATTT  294

Query  218   TGCCATTATCTTCATGTTAATACTTCAGATCAGAATACAATTATCTGAAGAAAGTGAATT  277
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295   TGCCATTATCTTCATGTTAATACTTCAGATCAGAATACAATTATCTGAAGAAAGTGAATT  354

Query  278   TTTAGTTGATAGGTCAAAAAACGGTCTCATCCACGTTCCTAAAGACCTATCCCAGAAAAC  337
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355   TTTAGTTGATAGGTCAAAAAACGGTCTCATCCACGTTCCTAAAGACCTATCCCAGAAAAC  414

Query  338   AACAATCTTAAATATATCGCAAAATTATATATCTGAGCTTTGGACTTCTGACATCTTATC  397
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415   AACAATCTTAAATATATCGCAAAATTATATATCTGAGCTTTGGACTTCTGACATCTTATC  474

Query  398   ACTGTCAAAACTGAGGATTTTGATAATTTCTCATAATAGAATCCAGTATCTTGATATCAG  457
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475   ACTGTCAAAACTGAGGATTTTGATAATTTCTCATAATAGAATCCAGTATCTTGATATCAG  534
```

```
Query  458   TGTTTTCAAATTCAACCAGGAATTGGAATACTTGGATTTGTCCCACAACAAGTTGGTGAA  517
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535   TGTTTTCAAATTCAACCAGGAATTGGAATACTTGGATTTGTCCCACAACAAGTTGGTGAA  594

Query  518   GATTTCTTGCCACCCTACTGTGAACCTCAAGCACTTGGACCTGTCATTTAATGCATTTGA  577
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595   GATTTCTTGCCACCCTACTGTGAACCTCAAGCACTTGGACCTGTCATTTAATGCATTTGA  654

Query  578   TGCCCTGCCTATATGCAAAGAGTTTGGCAATATGTCTCAACTAAAATTTCTGGGGTTGAG  637
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  655   TGCCCTGCCTATATGCAAAGAGTTTGGCAATATGTCTCAACTAAAATTTCTGGGGTTGAG  714

Query  638   CACCACACACTTAGAAAAATCTAGTGTGCTGCCAATTGCTCATTTGAATATCAGCAAGGT  697
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715   CACCACACACTTAGAAAAATCTAGTGTGCTGCCAATTGCTCATTTGAATATCAGCAAGGT  774

Query  698   CTTGCTGGTCTTAGGAGAGACTTATGGGGAAAAAGAAGACCCTGAGGGCCTTCAAGACTT  757
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775   CTTGCTGGTCTTAGGAGAGACTTATGGGGAAAAAGAAGACCCTGAGGGCCTTCAAGACTT  834

Query  758   TAACACTGAGAGTCTGCACATTGTGTTCCCCACAAACAAAGAATTCCATTTTATTTTGGA  817
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835   TAACACTGAGAGTCTGCACATTGTGTTCCCCACAAACAAAGAATTCCATTTTATTTTGGA  894

Query  818   TGTGTCAGTCAAGACTGTAGCAAATCTGGAACTATCTAATATCAAATGTGTGCTAGAAGA  877
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895   TGTGTCAGTCAAGACTGTAGCAAATCTGGAACTATCTAATATCAAATGTGTGCTAGAAGA  954

Query  878   TAACAAATGTTCTTACTTCCTAAGTATTCTGGCGAAACTTCAAACAAATCCAAAGTTATC  937
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  955   TAACAAATGTTCTTACTTCCTAAGTATTCTGGCGAAACTTCAAACAAATCCAAAGTTATC  1014

Query  938   AAATCTTACCTTAAACAACATTGAAACAACTTGGAATTCTTTCATTAGGATCCTCCAGCT  997
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  AAATCTTACCTTAAACAACATTGAAACAACTTGGAATTCTTTCATTAGGATCCTCCAGCT  1074

Query  998   GGTTTGGCATACAACTGTATGGTATTTCTCAATTTCAAACGTGAAGCTACAGGGTCAGCT  1057
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  GGTTTGGCATACAACTGTATGGTATTTCTCAATTTCAAACGTGAAGCTACAGGGTCAGCT  1134
```

```
Query  1058  GGACTTCAGAGATTTTGATTATTCTGGCACTTCCTTGAAGGCCTTGTCTATACACCAAGT  1117
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  GGACTTCAGAGATTTTGATTATTCTGGCACTTCCTTGAAGGCCTTGTCTATACACCAAGT  1194

Query  1118  TGTCAGCGATGTGTTCGGTTTTCCGCAAAGTTATATCTATGAAATCTTTTCGAATATGAA  1177
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  TGTCAGCGATGTGTTCGGTTTTCCGCAAAGTTATATCTATGAAATCTTTTCGAATATGAA  1254

Query  1178  CATCAAAAATTTCACAGTGTCTGGTACACGCATGGTCCACATGCTTTGCCCATCCAAAAT  1237
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  CATCAAAAATTTCACAGTGTCTGGTACACGCATGGTCCACATGCTTTGCCCATCCAAAAT  1314

Query  1238  TAGCCCGTTCCTGCATTTGGATTTTTCCAATAATCTCTTAACAGACACGGTTTTTGAAAA  1297
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  TAGCCCGTTCCTGCATTTGGATTTTTCCAATAATCTCTTAACAGACACGGTTTTTGAAAA  1374

Query  1298  TTGTGGGCACCTTACTGAGTTGGAGACACTTATTTTACAAATGAATCAATTAAAAGAACT  1357
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  TTGTGGGCACCTTACTGAGTTGGAGACACTTATTTTACAAATGAATCAATTAAAAGAACT  1434

Query  1358  TTCAAAAATAGCTGAAATGACTACACAGATGAAGTCTCTGCAACAATTGGATATTAGCCA  1417
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  TTCAAAAATAGCTGAAATGACTACACAGATGAAGTCTCTGCAACAATTGGATATTAGCCA  1494

Query  1418  GAATTCTGTAAGCTATGATGAAAAGAAAGGAGACTGTTCTTGGACTAAAAGTTTATTAAG  1477
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1495  GAATTCTGTAAGCTATGATGAAAAGAAAGGAGACTGTTCTTGGACTAAAAGTTTATTAAG  1554

Query  1478  TTTAAATATGTCTTCAAATATACTTACTGACACTATTTTCAGATGTTTACCTCCCAGGAT  1537
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1555  TTTAAATATGTCTTCAAATATACTTACTGACACTATTTTCAGATGTTTACCTCCCAGGAT  1614

Query  1538  CAAGGTACTTGATCTTCACAGCAATAAAATAAAGAGCATTCCTAAACAAGTCGTAAAACT  1597
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1615  CAAGGTACTTGATCTTCACAGCAATAAAATAAAGAGCATTCCTAAACAAGTCGTAAAACT  1674

Query  1598  GGAAGCTTTGCAAGAACTCAATGTTGCTTTCAATTCTTTAACTGACCTTCCTGGATGTGG  1657
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1675  GGAAGCTTTGCAAGAACTCAATGTTGCTTTCAATTCTTTAACTGACCTTCCTGGATGTGG  1734
```

```
Query  1658  CAGCTTTAGCAGCCTTTCTGTATTGATCATTGATCACAATTCAGTTTCCCACCCATCGGC  1717
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1735  CAGCTTTAGCAGCCTTTCTGTATTGATCATTGATCACAATTCAGTTTCCCACCCATCGGC  1794

Query  1718  TGATTTCTTCCAGAGCTGCCAGAAGATGAGGTCAATAAAAGCAGGGGACAATCCATTCCA  1777
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1795  TGATTTCTTCCAGAGCTGCCAGAAGATGAGGTCAATAAAAGCAGGGGACAATCCATTCCA  1854

Query  1778  ATGTACCTGTGAGCTAGGAGAATTTGTCAAAAATATAGACCAAGTATCAAGTGAAGTGTT  1837
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1855  ATGTACCTGTGAGCTAGGAGAATTTGTCAAAAATATAGACCAAGTATCAAGTGAAGTGTT  1914

Query  1838  AGAGGGCTGGCCTGATTCTTATAAGTGTGACTACCCGGAAAGTTATAGAGGAACCCTACT  1897
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1915  AGAGGGCTGGCCTGATTCTTATAAGTGTGACTACCCGGAAAGTTATAGAGGAACCCTACT  1974

Query  1898  AAAGGACTTTCACATGTCTGAATTATCCTGCAACATAACTCTGCTGATCGTCACCATCGT  1957
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1975  AAAGGACTTTCACATGTCTGAATTATCCTGCAACATAACTCTGCTGATCGTCACCATCGT  2034

Query  1958  TGCCACCATGCTGGTGTTGGCTGTGACTGTGACCTCCCTCTGCAGCTACTTGGATCTGCC  2017
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2035  TGCCACCATGCTGGTGTTGGCTGTGACTGTGACCTCCCTCTGCAGCTACTTGGATCTGCC  2094

Query  2018  CTGGTATCTCAGGATGGTGTGCCAGTGGACCCAGACCCGGCGCAGGGCCAGGAACATACC  2077
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2095  CTGGTATCTCAGGATGGTGTGCCAGTGGACCCAGACCCGGCGCAGGGCCAGGAACATACC  2154

Query  2078  CTTAGAAGAACTCCAAAGAAATCTCCAGTTTCATGCATTTATTTCATATAGTGGGCACGA  2137
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2155  CTTAGAAGAACTCCAAAGAAATCTCCAGTTTCATGCATTTATTTCATATAGTGGGCACGA  2214

Query  2138  TTCTTTCTGGGTGAAGAATGAATTATTGCCAAACCTAGAGAAAGAAGGTATGCAGATTTG  2197
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2215  TTCTTTCTGGGTGAAGAATGAATTATTGCCAAACCTAGAGAAAGAAGGTATGCAGATTTG  2274

Query  2198  CCTTCATGAGAGAAACTTTGTTCCTGGCAAGAGCATTGTGGAAAATATCATCACCTGCAT  2257
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2275  CCTTCATGAGAGAAACTTTGTTCCTGGCAAGAGCATTGTGGAAAATATCATCACCTGCAT  2334
```

```
Query  2258  TGAGAAGAGTTACAAGTCCATCTTTGTTTTGTCTCCCAACTTTGTCCAGAGTGAATGGTG  2317
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2335  TGAGAAGAGTTACAAGTCCATCTTTGTTTTGTCTCCCAACTTTGTCCAGAGTGAATGGTG  2394

Query  2318  CCATTATGAACTCTACTTTGCCCATCACAATCTCTTTCATGAAGGATCTAATAGCTTAAT  2377
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2395  CCATTATGAACTCTACTTTGCCCATCACAATCTCTTTCATGAAGGATCTAATAGCTTAAT  2454

Query  2378  CCTGATCTTGCTGGAACCCATTCCGCAGTACTCCATTCCTAGCAGTTATCACAAGCTCAA  2437
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2455  CCTGATCTTGCTGGAACCCATTCCGCAGTACTCCATTCCTAGCAGTTATCACAAGCTCAA  2514

Query  2438  AAGTCTCATGGCCAGGAGGACTTATTTGGAATGGCCCAAGGAAAAGAGCAAACGTGGCCT  2497
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2515  AAGTCTCATGGCCAGGAGGACTTATTTGGAATGGCCCAAGGAAAAGAGCAAACGTGGCCT  2574

Query  2498  TTTTTGGGCTAACTTAAGGGCAGCCATTAATATTAAGCTGACAGAGCAAGCAAAGAAATA  2557
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2575  TTTTTGGGCTAACTTAAGGGCAGCCATTAATATTAAGCTGACAGAGCAAGCAAAGAAATA  2634

Query  2558  GATTACACATCAAGTGAAAAATATTCCTCCTGTTGATATTGCTGCTTTTGGAAGTTCCAA  2617
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2635  GATTACACATCAAGTGAAAAATATTCCTCCTGTTGATATTGCTGCTTTTGGAAGTTCCAA  2694

Query  2618  CAATGACTTTATTTTGCATCAGCATAGATGTAAACACAATTGTGAGTGTATGATGTAGGT  2677
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2695  CAATGACTTTATTTTGCATCAGCATAGATGTAAACACAATTGTGAGTGTATGATGTAGGT  2754

Query  2678  AAAAAT  2683
              ||||||
Sbjct  2755  AAAAAT  2760
```